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13. ABSTRACT ***(Maximum 200 Words)***

Major insights into the molecular mechanisms of cancer have been obtained by studies of a family of regulatory molecules known as protein kinases. Many protein kinases serve as relays for signals in the cell that regulate normal growth and cellular function. In addition, several members of this family of molecules have previously been shown to be involved in the development of breast cancer in humans. We have cloned two novel protein kinases, *Hunk* and *Pnck*, that are turned on in the breast during specific stages of pregnancy, and that appear to be turned on to different degrees in different subgroups of breast cancer. Our studies have demonstrated that HUNK and PNCK are expressed in subsets of human breast cancers, that HUNK expression is downregulated in a large subset of human primary breast cancers, and that PNCK expression is upregulated in a large subset of human primary breast cancers. Our findings represent the first data implicating either a SNF1-related kinase or a CaM kinase in mammary carcinogenesis.

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1. Chodosh et al. Protein kinase expression during murine mammary gland development. *Developmental Biology,* 219:259-276, 2000.

2. Gardner et al.. Cloning and characterization of *Hunk,* a novel mammalian SNF1-related protein kinase. *Genomics* 63:46-59, 2000.

3. Gardner et al. Cloning, characterization, and chromosomal localization of *Pnck*, a calcium/calmodulin-dependent protein kinase. *Genomics* 63:279-288, 2000.

4. Gardner et al. Developmental role of the SNF1-related kinase Hunk in pregnancy-induced changes in the mammary gland. *Development* 127:4493-4509, 2000.

5. Gardner et al. The CaM kinase, *Pnck*, is spatially and temporally regulated during murine mammary gland development and may identify an epithelial cell subtype involved in breast cancer. *Cancer Research*, 60:5571-5577, 2000.

INTRODUCTION

Major insights into the molecular mechanisms of cancer have been obtained by studies of the protein kinase family of regulatory molecules. Many protein kinases serve as relays for signals in the cell that regulate normal growth and cellular function. In addition, several members of this family of molecules have previously been shown to be involved in the development of breast cancer in humans. Indeed, the increased activity of some of these molecules has been shown to correlate with aggressive tumor behavior and poor clinical outcome in women with breast cancer. We have cloned two novel protein kinases, *Hunk* and *Pnck* (formerly called *Punc*), that are turned on in the breast during specific stages of pregnancy, and that appear to be turned on to different degrees in different subgroups of breast cancer. Our observations suggest that these genes may represent valuable biological markers in diagnosing cancer, in predicting the biological behavior of breast cancer, or in understanding the causes of breast cancer in humans. As such, we believe that continued study of these molecules may yield novel insights into those cell types in the breast that are most susceptible to carcinogenesis, and into those pathways in the cell that regulate growth.

BODY

SPECIFIC AIMS

I. Determine the Basis for Differential *Hunk* and *Pnck* Expression in *neu*- and *c-Myc*-initiated Breast Cancers.

Different oncogenes may give rise to different tumor types either because: (1) distinct cell types exist within the breast that are preferentially susceptible to particular types of initiating events (i.e. *neu* vs. *c-myc*); (2) there is one shared target cell type for transformation in the breast that is driven into distinct pathways by the action of different oncogenes.

II. Determine Whether *HUNK* and *PNCK* are Amplified, Overexpressed or Mutated in Human Breast Cancers

Protein kinases function as oncogenes in a variety of human cancers. The finding that *HUNK* and/or *PNCK* are overexpressed or mutated in human breast cancers would imply that these molecules play an important role in human carcinogenesis. Therefore, we will examine the status of the human *HUNK* and *PNCK* genes in a panel of human breast cancer cell lines, and in a series of primary human breast cancers.

TECHNICAL OBJECTIVES

Technical Objective I: Investigate the mechanism for the differential expression of *Hunk* and *Pnck* in *neu* and *c-myc*-initiated breast cancers.

Task 1: Months 1-12: Generate antisera specific for Hunk and Pnck .

Task 2: Months 1-24: Determine the basis for differential expression of *Hunk* and *Pnck* in transgenic mice.

Task 3: Months 1-24: Determine the basis for differential expression of *Hunk* and *Pnck* in transgenic breast cancer cell lines.

Technical Objective II: Determine whether *HUNK* and *PNCK* are amplified, overexpressed or mutated in human breast cancers.

Task 1: Months 6-24: Determine whether *HUNK* or *PNCK* are amplified, overexpressed or mutated in human breast cancer cell lines.

Task 2: Months 6-24: Determine whether *HUNK* or *PNCK* are amplified, overexpressed or mutated in primary human breast cancers.

Please note that the results from this study are described in detail in the five accompanying manuscripts that have been supported by this grant. These manuscripts are:

1. Chodosh LA*, Gardner HP, Rajan JV, Stairs DB, Marquis ST, and Leder P. Protein kinase expression during murine mammary gland development. *Developmental Biology,* 219:259-276, 2000. (*corresponding author)

2. Gardner HP, Wertheim GB, Ha SI, Copeland NG, Gilbert DJ, Jenkins NA, Marquis ST, and Chodosh LA. Cloning and characterization of *Hunk,* a novel mammalian SNF1-related protein kinase. *Genomics* 63:46-59, 2000.

3. Gardner HP, Rajan JV, Ha SI, Copeland NG, Gilbert DJ, Jenkins NA, Marquis ST and Chodosh LA. Cloning, characterization, and chromosomal localization of *Pnck*, a calcium/calmodulin-dependent protein kinase. *Genomics* 63:279-288, 2000.

4. Gardner HP, Belka GK, Wertheim GBW, Hartman JL, Ha SI, Gimotty PA, Marquis ST, and Chodosh LA. Developmental role of the SNF1-related kinase Hunk in pregnancy-induced changes in the mammary gland. *Development* 127:4493-4509, 2000.

5. Gardner HP, Ha SI, Reynolds C, and Chodosh LA. The CaM kinase, *Pnck*, is spatially and temporally regulated during murine mammary gland development and may identify an epithelial cell subtype involved in breast cancer. *Cancer Research*, 60:5571-5577, 2000.

Please refer to these manuscripts for details not included in the present report.

Technical Objective I: Investigate the mechanism for the differential expression of *Hunk* and *Pnck* in *neu* and *c-myc*-initiated breast cancers.

Task 1: Months 1-12: Generate antiseral specific for Hunk and Pnck .

This task has been completed on schedule. Since understanding the function of *Hunk* and *Pnck* will require the ability to reliably detect the proteins encoded by these genes and the activities associated with them, we generated antisera specific for mouse *Hunk* and *Pnck*. For each kinase, glutathione-S-transferase (GST) fusion protein constructs were generated that encode three non-overlapping regions of each molecule. Recombinant GST fusion proteins were produced in protease-deficient *E. coli*, purified over glutathione-sepharose columns, and cleaved into separate GST and kinase polypeptide domains. Each purified polypeptide was injected into two rabbits. All injections, prebleeds and bleeds were performed by an off-site vendor (Colcalico, Inc) using standard methods. The specificity of affinity-

purified antisera was assessed by immunoblotting against endogenous as well as exogenously overexpressed *Hunk* and *Pnck* from unlabeled whole-cell lysates followed by immunoblotting using

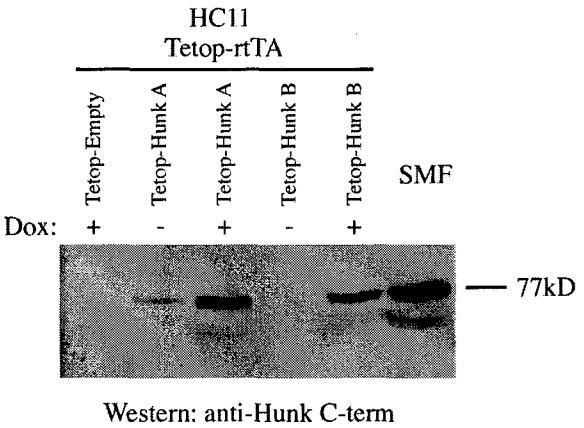

Figure 1: Detection of Hunk protein exogenously expressed in HC11 mammary epithelial cells

antisera raised against different regions of these molecules. As an example, studies with Hunk are shown. We initially demonstrated that anti-Hunk antibodies would recognize exogenously expressed Hunk either in SMF cells (a mammary epithelial cell line isolated from an MMTV-neu transgenic mouse mammary tumor) or in HC11 mammary epithelial cells that we engineered to inducibly express Hunk when treated with doxycycline (Fig. 1).

Next, we demonstrated that anti-Hunk antibodies raised against distinct regions of the Hunk molecule could identify Hunk that was consitutively overexpressed in the mammary glands of MMTV-Hunk transgenic mice (MHK3). Hunk was immunoprecipitated from mammary gland extracts of either wild-type (Wt) or MHK3 transgenic animals (Tg) using an antibody directed against the carboxyl-terminus of Hunk, and then was detected by western immunoblotting using anti-Hunk antibodies (Fig. 2A). These results revealed detection of Hunk only in the mammary glands of Hunk-overexpressing transgenic animals. Immunoprecipitated Hunk was shown to have kinase activity by incubating anti-Hunk immunoprecipitates with ^{32}P-ATP and histone as substrate (Fig. 2B). These antibodies were then shown to specifically recognize Hunk in the mammary glands of MHK3 transgenic animals by performing immunohistochemical studies using these anti-Hunk antibodies (Fig. 2C).

To demonstrate that the anti-Hunk antibodies that we raised were able to detect endogenously expressed Hunk, we performed immunoblotting of immunoprecipitates from protein extracts of lung tissue harvested either from wild-type animals, or animals in which we have deleted the *Hunk* locus by homologous recombination. These studies clearly show Hunk protein of the expected size in wild-type animals but not in animals in which the *Hunk* locus has been deleted (Fig. 3). Interestingly, heterozygous animals exhibit steady-state levels of Hunk protein expression that are approximately half of that observed in wild-type mice, indicating that there is no dosage-compensation for loss of one *Hunk* allele.

Finally, to determine whether the developmental pattern of Hunk expression that we previously identified based on RNA expression data correctly predicted protein expression profiles, we performed IP/Westerns on mammary gland lysates to measure Hunk protein expression levels during postnatal mammary development (Fig. 4). These studies clearly demonstrated the specific upregulation of Hunk protein expression during early pregnancy that we previously described at the mRNA level.

In summary, our ability to use multiple antisera to detect the same protein, our ability to demonstrate that the proteins detected by these antisera are upregulated in cells and tissues

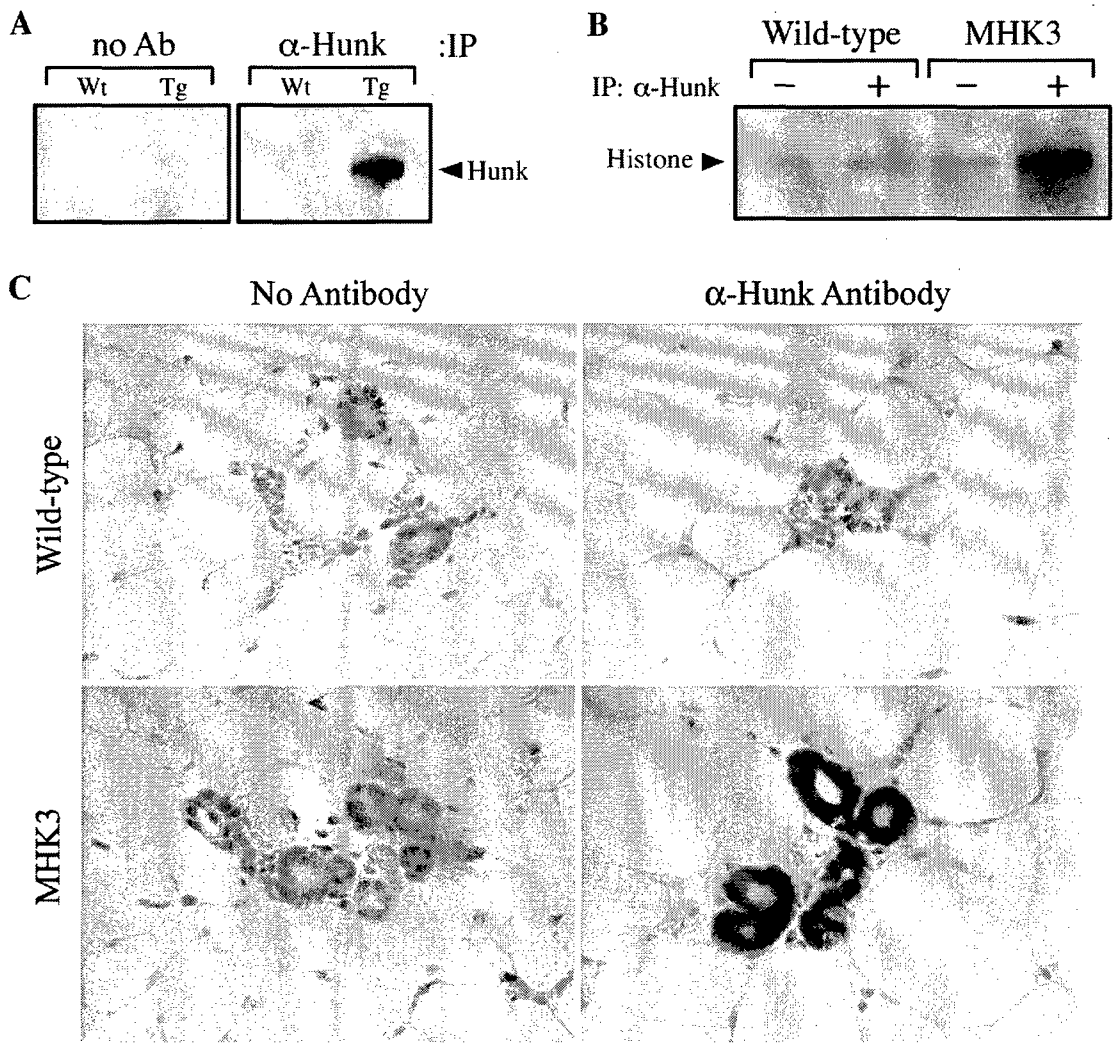

Figure 2: Detection of Hunk protein exogenously expressed in transgenic mice

overexpressing these kinases, and our ability to demonstrate that the proteins detected by these antisera are absent in tissues harvested from animals in which Hunk has been deleted, provide strong evidence that the antisera that we have generated are able to identify the protein products of the *Hunk* and *Pnck* genes.

Task 2: Months 1-24: Determine the basis for differential expression of *Hunk* and *Pnck* in transgenic mice.

This task has been completed on schedule. The differential expression of *Hunk* and *Pnck* in mammary epithelial cell lines derived from tumors arising in *neu* and *myc* transgenic mice may be a direct result of the differential induction of these kinases by the overexpression of *neu*- or *c-myc* in the non-transformed mammary epithelium. Alternately, this pattern of differential expression could result if the overexpression of *Hunk* (or *Pnck*) in *neu*-induced (or *c-myc*-induced) tumors reflects the selection and outgrowth of a $Hunk^+$ (or $Pnck^+$) epithelial cell subtype that otherwise represents a minor fraction of cells in the mammary epithelium. In this case, differential patterns of *Hunk* and *Pnck* expression would only expect to be seen in mammary tumors and not in intact mammary glands. To distinguish

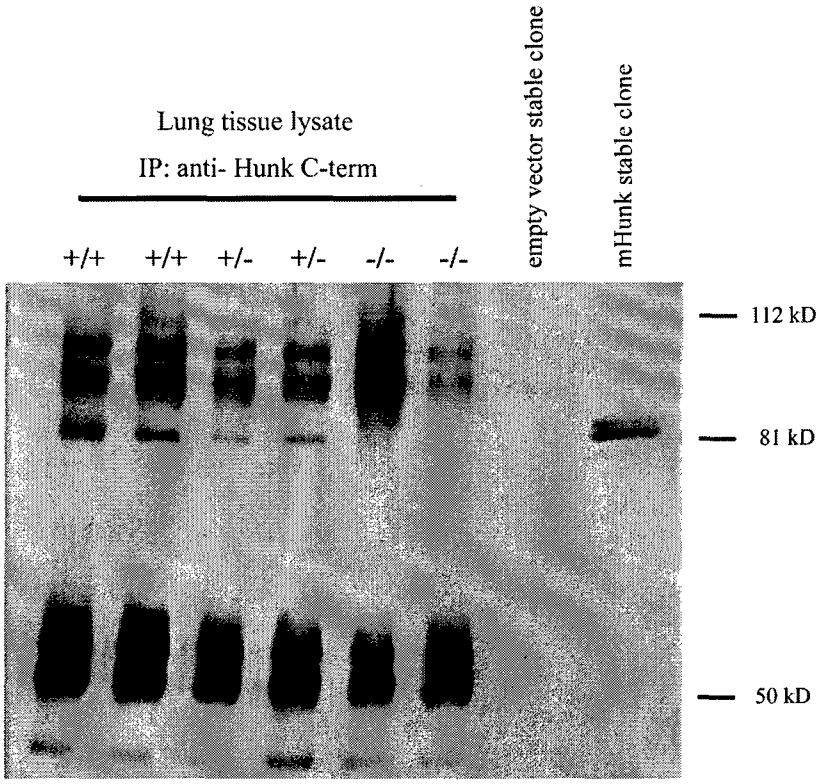

Figure 3: Detection of Hunk protein in wild-type, but not *Hunk*-deleted, mice

between these possibilities, nontransformed mammary gland tissue was harvested from MMTV-*neu* and MMTV-*c-myc* transgenic mice prior to the appearance of tumors and RNA was prepared from snap-frozen mammary glands. Mammary gland whole mounts and representative H&E sections were also analyzed to confirm the absence of tumors. *Hunk* and *Pnck* mRNA expression levels were determined by RNase protection in nontransformed *neu* and *c-myc* transgenic breast tissue, as well as in breast tissue from nontransgenic littermates. In addition, breast tissue was also harvested from virgin animals and from animals during early and late stages of pregnancy (when *c-myc* and *neu* transgene expression is highest) and was also assayed for *Hunk* and *Pnck* expression.

This analysis revealed that *Hunk* and *Pnck* mRNA expression levels in MMTV-neu and MMTV-c-myc transgenic animals are indistinguishable from those of wild-type animals during virgin development and during pregnancy and lactation. This finding suggests either: 1) that *Hunk* and *Pnck* expression are not induced either directly or indirectly by neu or c-myc expression in the mammary gland; 2) that MMTV-driven transgene expression of neu and c-myc in the non-tumorigenic mammary gland does not occur in cells that are capable of expressing Hunk or Pnck; or 3) that neu and c-myc are able to induce *Hunk* and/or *Pnck* expression in tumorigenic cells, but not in non-transformed cells.

To distinguish between these possibilities, we harvested mammary tumors arising in MMTV-neu and MMTV-c-myc transgenic animals. RNA was subsequently prepared from snap-frozen tumors and *Hunk* and *Pnck* mRNA expression levels were determined by RNase protection analysis. Surprisingly, these studies demonstrated that, unlike the cell lines derived from MMTV-neu and MMTV-c-myc mammary tumors, the tumors themselves did not exhibit differences in the steady-state expression levels of either *Hunk* or *Pnck*. This suggested that the differential pattern of kinase expression that was

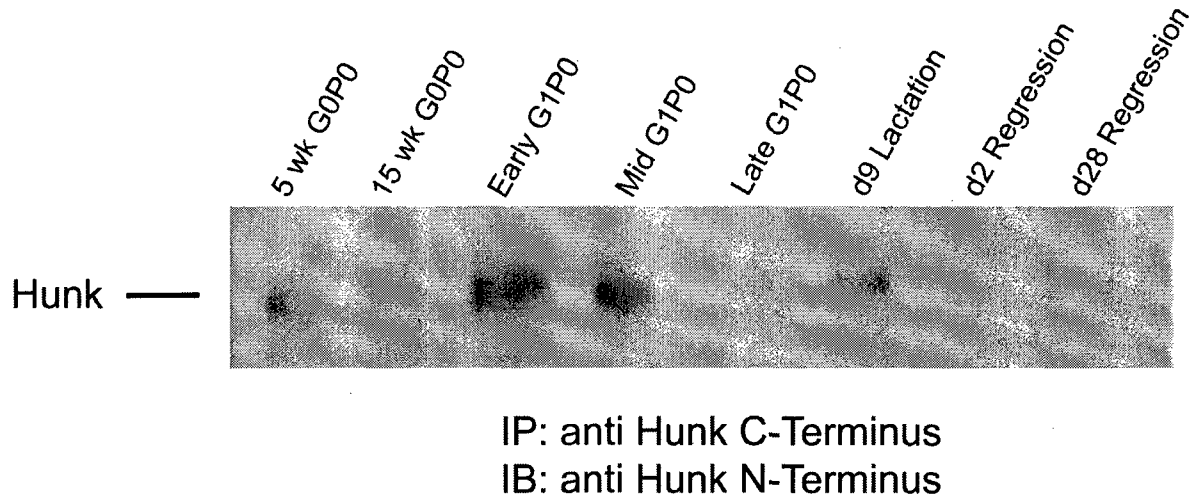

Figure 4: Detection of Hunk protein during mammary gland development

observed in the transgenic mammary epithelial cell lines obtained from Dr. Philip Leder's laboratory is not a reflection of differential kinase expression in the tumors from which they were derived. Alternately, we hypothesized that the differential pattern of Hunk and Pnck expression might only be manifest under *in vitro* culture conditions.

To test this possibility, we generated 10 mammary epithelial cell lines from MMTV-neu-induced mammary tumors and 10 mammary epithelial cell lines from MMTV-c-myc-induced mammary tumors. These cell lines were grown in culture and RNA was harvested from cell pellets and assayed for steady-state levels of *Hunk* and *Pnck* mRNA expression. Surprisingly, similar to the primary tumors from which they were derived, the neu and myc-induced mammary tumor cell lines that we generated also did not display differential expression of either *Hunk* or *Pnck*. Our results suggest that the differential pattern of *Hunk* and *Pnck* expression that we repeatedly observe in the neu and myc-induced mammary tumor cell lines obtained from the Leder laboratory is more likely to be due to differences in the manner in which the neu and myc cell lines were generated, than to intrinsic differences in *Hunk* or *Pnck* expression in cell lines derived from neu and myc-induced tumors or in the mammary tumors themselves. As such, we favor the possibility that different culture conditions were employed to isolate the neu-induced tumor cell lines than were used to isolate the myc-induced tumor cell lines. Unfortunately, since these cell lines were generated more than 12 years ago, it is impossible to formally prove this hypothesis.

Task 3: Months 1-24: Determine the basis for differential expression of *Hunk* and *Pnck* in transgenic breast cancer cell lines.

The model that a single target cell for transformation can be driven down different pathways by *neu* or *c-myc* to yield tumors with different phenotypes predicts that genes that are preferentially expressed in *neu*-initiated tumors (i.e. *Hunk*) are either up-regulated by the *neu* signal transduction pathway or are down-regulated by the *c-myc* transduction pathway. Conversely, the model that Hunk-positive and Pnck-negative cell lines are preferentially transformed by neu, whereas Hunk-negative and Pnck-positive cell lines are preferentially transformed by myc, predicts that cell lines derived from neu-induced tumors and from myc-induced tumors represent different cell types. Our findings above

strongly argued against the first model, since we did not observe differential expression in neu and myc-induced tumors or cell lines. In fact, our observations above also argued against the second model. As such, we concluded that the experimental approach that was originally proposed in this task was no longer valid. Instead, we reasoned that differences in Hunk and Pnck gene expression patterns among the neu and myc transgenic breast cancer cell lines originally isolated in the Leder laboratory might reflect the outgrowth of different epithelial cell types under different culture conditions. Accordingly, we hypothesized that we might be able to detect such differences in cell types by comparing global gene expression profiles in these original Hunk-positive/Pnck-negative and Hunk-negative/Pnck-positive cell lines.

Standard Affymetrix protocols were used to generate and hybridize probes prepared from RNA samples from each of 9 Hunk-positive/Pnck-negative and 9 Hunk-negative/Pnck-positive cell lines. RNA isolated from these cell lines was subject to reverse transcription to make first strand cDNA that incorporated a T7 RNA polymerase promoter at the 3' end. Following completion of the second strand of cDNA, this material was used in an in vitro transcription reaction to generate biotin-labeled cRNA representing each experimental pool of mammary gland RNA. This in vitro transcribed cRNA was then fragmented to facilitate hybridization. Hybridization reactions were prepared for each sample using fragmented cRNA in addition to standardized internal controls. Hybridization reactions representing each of the 18 cell line samples were hybridized to murine U74A oligonucleotide microarrays, representing approximately 12,000 murine genes.

Using GeneChip software, expression levels were calculated for each of the 12,000 genes represented on the U74A chips for all 18 samples. This information was imported into a software database. Confidence intervals were generated for each gene in order to identify genes that were differentially expressed between Hunk-positive/Pnck-negative and Hunk-negative/Pnck-positive cell lines. Our results reveal the differential expression of approximately 600 genes between these two groups of cell lines. In-depth analysis of these data sets are currently underway.

Technical Objective II: Determine whether *HUNK* and *PNCK* are amplified, overexpressed or mutated in human breast cancers.

Task 1: Months 6-24: Determine whether *HUNK* or *PNCK* are amplified, overexpressed or mutated in human breast cancer cell lines.

This task has been completed on schedule. Protein kinases function as oncogenes in a variety of human cancers. The finding that *HUNK* and/or *PNCK* are overexpressed or mutated in human breast

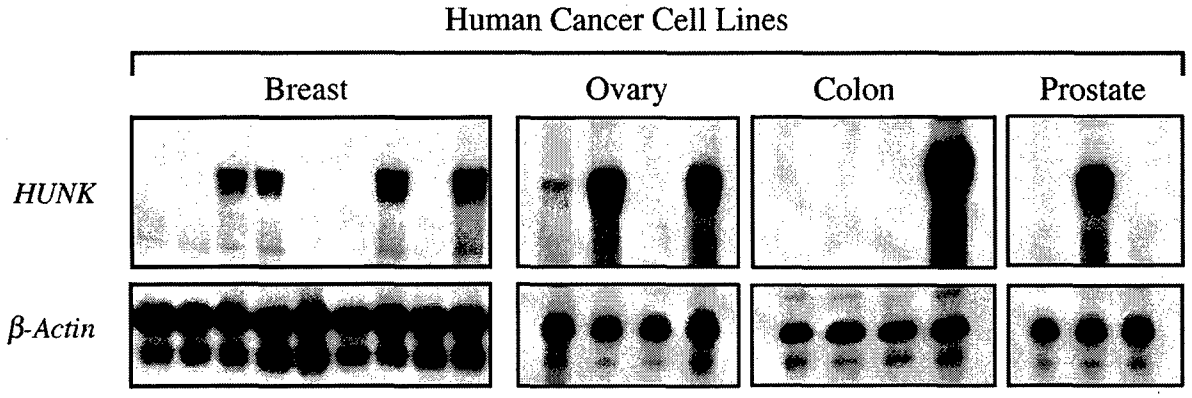

Figure 5: *HUNK* expression in human cancer cell lines

cancers would imply that these molecules play an important role in human carcinogenesis. Therefore, we examined the status of the human *HUNK* and *PNCK* genes in a panel of human breast cancer cell lines as well as cell lines representing other common types of human cancers. Genomic DNA and total cellular RNA were prepared from human cancer cell lines obtained from ATCC and other commercial sources and were grown and harvested under similar conditions. To detect DNA amplifications and rearrangements, Southern blots containing genomic DNA harvested from each of these cell lines were probed with full-length human *HUNK* and *PNCK* cDNAs. Blots were subsequently reprobed with a cDNA for GAPDH as a control for equal DNA loading. These studies did not reveal any evidence for amplification of *HUNK* or *PNCK* in human cancer cell lines.

To detect alterations in *HUNK* and *PNCK* expression at the mRNA level, RNase protection analysis was performed from total RNA samples prepared from each of these cell lines. These studies revealed that *HUNK* and *PNCK* expression are highly heterogeneous in human cancer cell lines representing a variety of different types of cancer (Figs. 5-7). That is, within any one tumor type, cell lines can be found that express high levels of HUNK or PNCK, whereas the expression level of these kinases in other cell lines was low or undetectable. For example, analogous to results observed in murine cell lines, *HUNK* expression levels varied widely among human breast tumor cell lines as well as among cell lines derived from other types of cancers (Fig. 5). In particular, the range of *HUNK* expression observed in human breast tumor cell lines was comparable to that observed murine mammary tumor cell lines. Cell lines expressing *HUNK* at high levels, as well as cell lines with undetectable *HUNK* expression levels, were observed for all tumor types examined. For example, 3 of 4 colon tumor cell lines examined do not express detectable levels of *HUNK*, whereas one of these cell lines expresses *HUNK* at high levels. Similar results were observed for cell lines derived from tumors of the central nervous system, breast, lung, ovary, prostate, and kidney (Fig. 5 and data not shown). In contrast to results obtained in the murine mammary tumor cell lines, *HUNK* expression in human breast tumor cell lines does not appear to correlate with *HER2/NEU* expression (data not shown).

To further investigate the potential involvement of *Pnck,* or a cell type in which *Pnck* is expressed, in mammary carcinogenesis, we determined *PNCK* expression levels in a panel of human breast cancer cell lines (Fig. 6). Similar to the wide range of *Pnck* expression observed in the murine mammary epithelium and in murine mammary tumor cell lines, *PNCK* expression was detected in only a subset of human breast tumor cell lines. High levels of *PNCK* expression were observed in only 3 of 18 breast tumor cell lines. Eight cell lines expressed low, but detectable levels of *PNCK*, whereas no *PNCK*

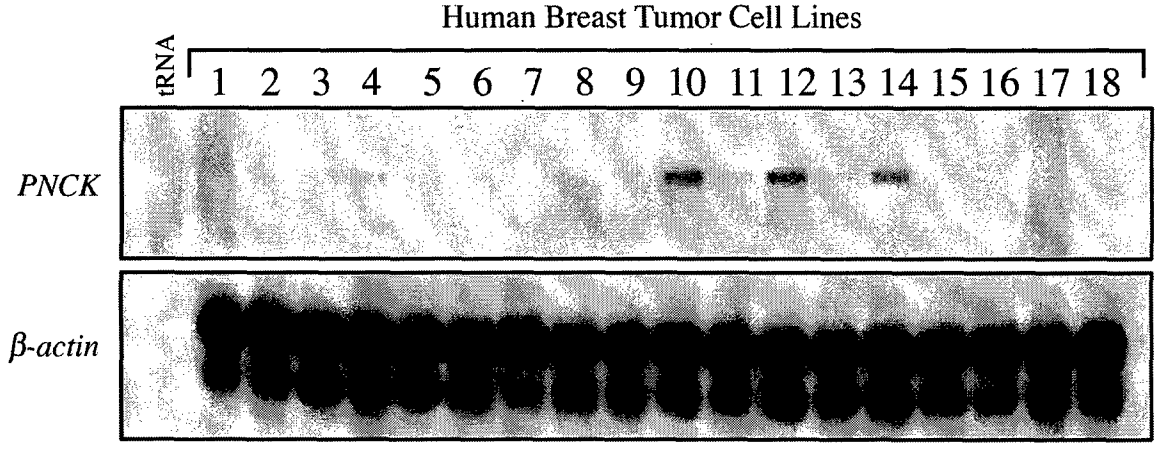

Figure 6: *PNCK* expression in human breast cancer cell lines

expression was detected in the remaining 7 cell lines. *PNCK* expression levels did not correlate with *c-MYC* expression (data not shown). To begin to examine the potential role of PNCK in other types of human cancers, we determined the pattern of *PNCK* expression in a panel of human tumor cell lines from multiple tissues of origin (Fig. 7). Similar to results observed in breast tumor cell lines, *PNCK* expression was heterogeneous among cell lines from every tumor type examined. Most striking was the observation that *PNCK* is expressed in 3 of the 4 small cell lung carcinoma cell lines, but is detected in only one of ten other lung cancer cell

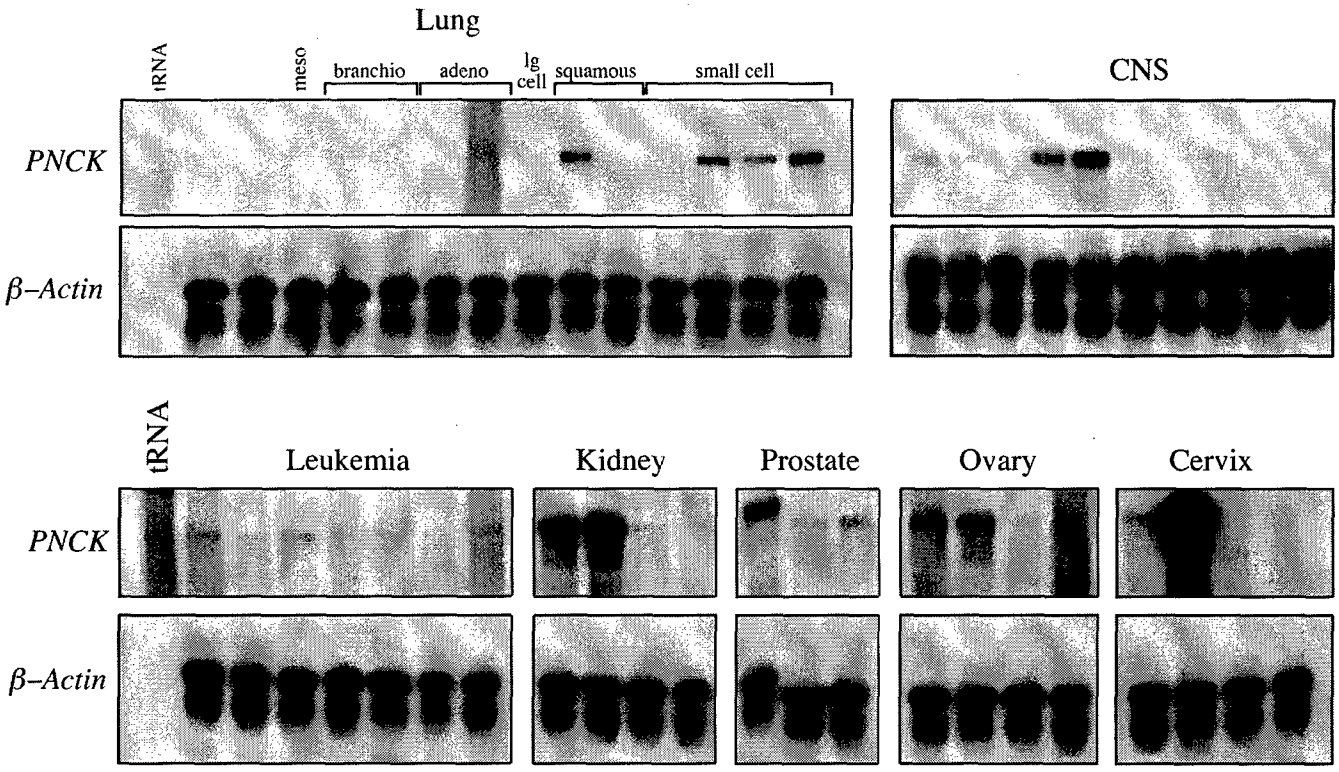

Figure 7: *PNCK* expression in human cancer cell lines

lines. This finding suggests that *PNCK* expression in lung cancers may be specific to small cell lung carcinomas. In the CNS, leukemia, kidney, prostate, ovary and cervical human tumor cell lines surveyed, relatively high levels of *PNCK* expression were observed in a subset of samples for each tumor type. These data suggest that heterogeneous *PNCK* expression is not limited to breast tumor cell lines, but rather is common to a variety of human cancers.

Task 2: Months 6-24: Determine whether *HUNK* or *PNCK* are amplified, overexpressed or mutated in primary human breast cancers.

This task has been completed on schedule. The high degree of heterogeneity in *HUNK* expression levels among murine and human tumor cell lines within a given tumor type was intriguing since it suggested the possibility that distinct subtypes of tumors exist that can be distinguished on the basis of high versus low levels of *HUNK* expression. However, the heterogeneity in *HUNK* expression levels observed among these cell lines could also reflect heterogeneous expression within the primary tumors from which these cell lines were derived, or could be a consequence of changes that occurred during the establishment or maintenance of these cell lines in culture. To begin to investigate the basis of the

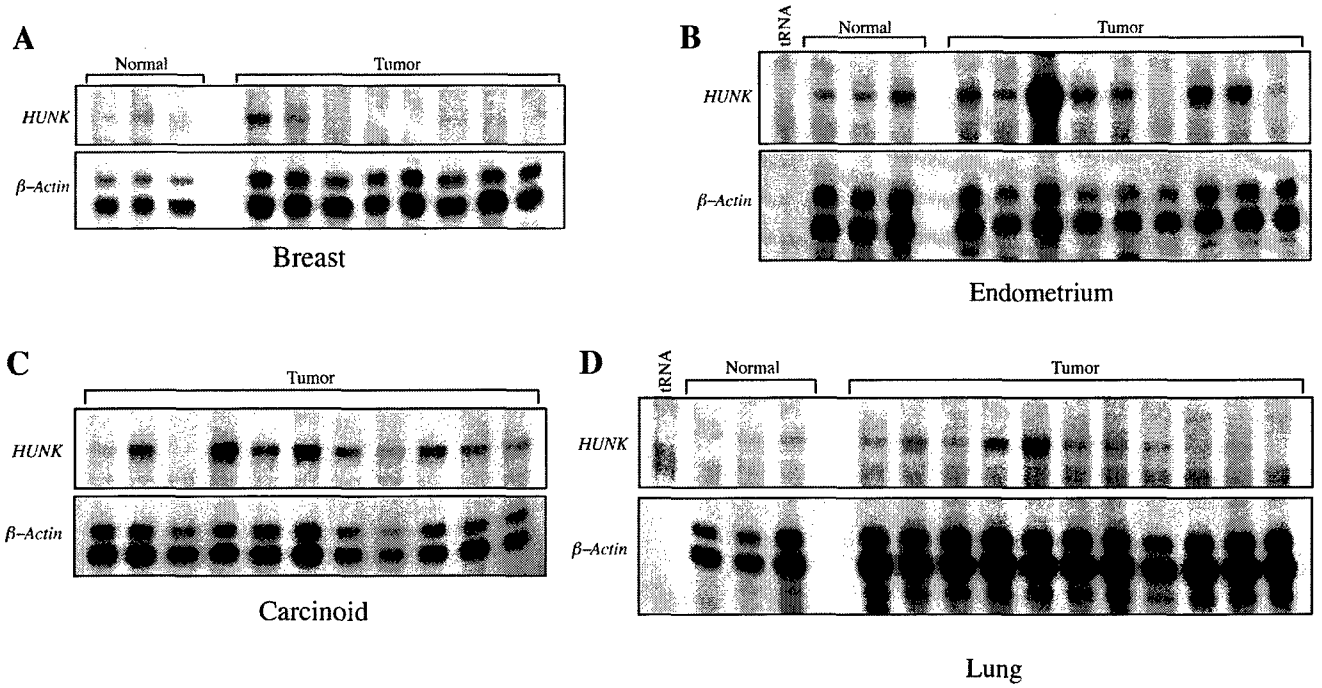

Figure 8: *HUNK* expression in primary human cancers

heterogeneity in *HUNK* expression observed among tumor cell lines, *HUNK* expression was examined in eight different types of primary human tumors and in benign tissues corresponding to a subset of these different tissue types. Consistent with the highly heterogeneous levels of *HUNK* expression observed in tumor cell lines, *HUNK* exhibited a wide dynamic range of expression among tumors in each of the tissue-types examined (Fig. 8 A-D). In contrast, *HUNK* expression levels in normal tissue samples were relatively similar within a given tissue. These data suggest that the highly heterogeneous levels of *HUNK* expression observed in tumor cell lines may reflect the existence of *HUNK*-expressing and *HUNK*-non-expressing tumors rather than an artifact of cell culture. In particular, HUNK expression in a large subset of human primary breast cancers was actually lower than that observed in normal breast tissue (Fig. 8A and data not shown). This is particularly evident when normalizing to cytokeratin 18 expression, since *HUNK* expression is restricted to the mammary epithelium (data not shown). We have confirmed this findings by quantitative real-time PCR of approximately 20 primary human breast cancers.

Similar to *HUNK*, the heterogeneous pattern of *PNCK* expression observed *in vitro* in both murine and human breast tumor cell lines suggested the possibility that *PNCK*-expressing and *PNCK* non-expressing breast tumor types might exist. In order to test this hypothesis directly we used RNase protection analysis to quantitate *PNCK mRNA* expression levels in a panel of 23 primary human breast tumors. The resulting expression levels were compared to *PNCK* expression levels in 12 benign breast tissue samples (Fig. 9A). This analysis revealed two interesting aspects of the pattern of *PNCK* expression in breast tumors compared to benign tissue: first, *PNCK* is expressed at significantly higher levels in breast tumors compared to benign tissue; and second, *PNCK* expression in human tumors is markedly heterogeneous.

Statistical analysis of the above *PNCK* expression levels indicated that when normalized to *β-actin* expression *PNCK* expression in human primary breast cancers is approximately 5-fold higher than in benign breast tissue (student's t-test, p=0.01; Fig. 9B). However, since PNCK expression in the mammary gland is epithelial specific, and since tumors typically have a higher epithelial content than benign breast tissue, we also normalized *PNCK* expression to the expression of the epithelial-specific

marker, *cytokeratin 18,* to control for the increased epithelial cell content of tumors (Fig. 9B). Strikingly, even after normalization to *CK18* expression, *PNCK* expression levels were found to be 3 times higher in human primary breast tumors than in benign tissue (t-test, p=0.039).

Formally, the increase in *PNCK* expression levels in breast tumors compared to benign tissue could result either from increased expression among all tumors or from increased expression in a subset of tumors. In this regard, analysis of the distribution of *PNCK* expression among the 23 ductal carcinomas

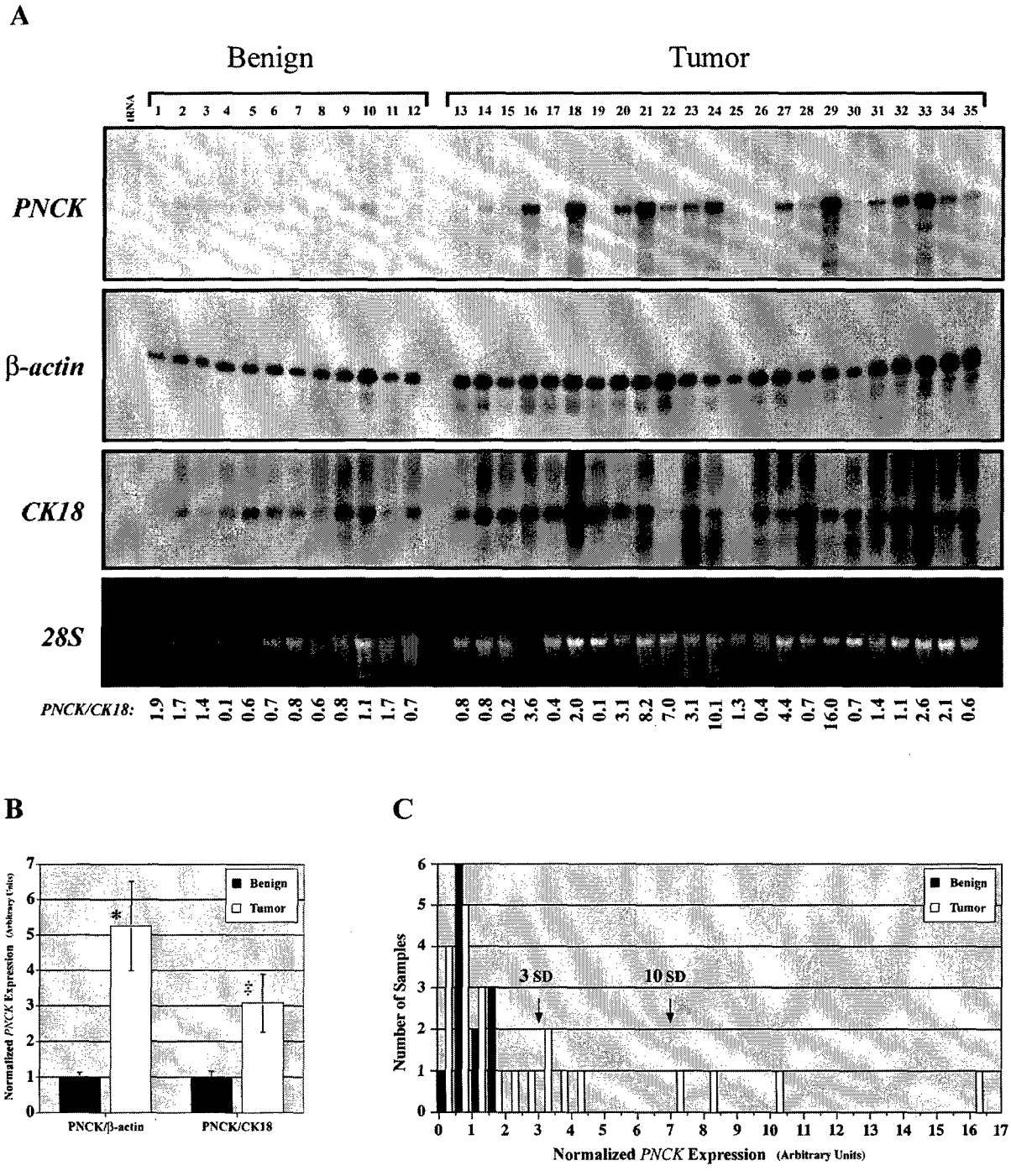

Figure 9: *PNCK* expression in primary human breast cancers

studied revealed a wide range of *PNCK* expression levels, in contrast to the relatively similar levels of *PNCK* expression observed among benign breast tissue samples, despite the fact the mode for the benign and tumor distributions was the same (Fig. 9A and 9C). Indeed, examination of the histogram representing *CK18*-normalized *PNCK* expression levels revealed that 8 of the 23 primary breast tumors analyzed express *PNCK* at levels greater than 3 standard deviations above the mean observed for benign samples (Fig. 6C). This difference is highly significant since no tumors would have been predicted to express *PNCK* at these levels if the distribution of *PNCK* expression in tumors was similar to that observed in benign tissues. Even more strikingly, four breast tumors were found to express *PNCK* at levels greater than 10 standard deviations above the mean observed for benign tissues. Together, these data indicate that PNCK is overexpressed in human primary breast cancers compared to benign tissue, and that this observed increase is due to high levels of *PNCK* expression in a subset of breast tumors.

KEY RESEARCH ACCOMPLISHMENTS

- Identification of Hunk and Pnck proteins in murine tissues and verification of developmental patterns of expression in the mammary gland.
- Demonstration that *HUNK* expression is restricted to a subset of human breast tumor cell lines.
- Demonstration that *HUNK* expression is downregulated in a subset human primary breast cancers compared to benign breast tissue.
- Demonstration that *PNCK* expression is restricted to a subset of human breast tumor cell lines.
- Demonstration that *PNCK* is overexpressed in human primary breast cancers compared to benign breast tissue and that this overexpression is restricted to a subset of human breast tumors . .

REPORTABLE OUTCOMES

Publication of 5 manuscripts supported by this grant:

1. Chodosh LA*, Gardner HP, Rajan JV, Stairs DB, Marquis ST, and Leder P. Protein kinase expression during murine mammary gland development. *Developmental Biology,* 219:259-276, 2000. (*corresponding author)

2. Gardner HP, Wertheim GB, Ha SI, Copeland NG, Gilbert DJ, Jenkins NA, Marquis ST, and Chodosh LA. Cloning and characterization of *Hunk,* a novel mammalian SNF1-related protein kinase. *Genomics* 63:46-59, 2000.

3. Gardner HP, Rajan JV, Ha SI, Copeland NG, Gilbert DJ, Jenkins NA, Marquis ST and Chodosh LA. Cloning, characterization, and chromosomal localization of *Pnck*, a calcium/calmodulin-dependent protein kinase. *Genomics* 63:279-288, 2000.

4. Gardner HP, Belka GK, Wertheim GBW, Hartman JL, Ha SI, Gimotty PA, Marquis ST, and Chodosh LA. Developmental role of the SNF1-related kinase Hunk in pregnancy-induced changes in the mammary gland. *Development* 127:4493-4509, 2000.

5. Gardner HP, Ha SI, Reynolds C, and Chodosh LA. The CaM kinase, *Pnck*, is spatially and temporally regulated during murine mammary gland development and may identify an epithelial cell subtype involved in breast cancer. *Cancer Research*, 60:5571-5577, 2000.

Publication of 2 abstracts supported by this grant:

1. Gardner HP, Ha SI, Rajan JV, and Chodosh LA. Novel kinases in mammary gland development and carcinogenesis. Endocrine Society '99. June, 1999. San Diego, CA

2. Gardner HP, Ha SI, Rajan JV, and Chodosh LA. Novel kinases in mammary gland development and carcinogenesis. Mammary Gland Biology Gordon Research Conference. June, 1999. Hennecker, NH

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CONCLUSIONS

Our initial observations of the oncogene-associated pattern of *Hunk* expression in murine mammary tumor cell lines along with the observation that *Hunk* expression affects mammary epithelial cell proliferation *in vivo*, suggested that *Hunk* may play a role in mammary carcinogenesis. We originally noted that in murine mammary tumor cell lines, *Hunk* is expressed in an oncogene-associated manner with high levels of expression observed in cell lines derived from MMTV-*neu* and MMTV-H-*ras* transgenic mice, but not in MMTV-c-*myc* or MMTV-*int-2/Fgf3* transgenic mice. To further investigate the role of Hunk in carcinogenesis, we examined the expression of human *HUNK* in human tumor cell lines and primary tumors from a variety of tissues including the breast. Similar to results observed in murine cell lines, *HUNK* expression levels varied widely among the human tumor cell lines and primary tumors examined in this study. In particular, our data further demonstrated that *HUNK* expression is downregulated in a subset of human primary breast cancers. Together with our previous findings that Hunk overexpression in the mammary gland inhibits epithelial proliferation, these data suggest a potential role for *HUNK* in human breast cancer.

Analogous to *HUNK*, expression of the human homologue of *Pnck* was found to be restricted to a subset of human breast tumor cell lines. Moreover, we have demonstrated that *PNCK* is overexpressed in human primary breast cancers compared to benign breast tissue and that this overexpression is restricted to a subset of human breast tumors. In aggregate, our findings are consistent with the hypothesis that *PNCK* expression is restricted to a subset of ductal carcinomas in humans, and suggest a role for *PNCK* or a cell type that expresses *PNCK* in mammary carcinogenesis. Our findings represent the first data implicating either a SNF1-related kinase or a CaM kinase in mammary carcinogenesis.

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Protein Kinase Expression during Murine Mammary Development

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The susceptibility of the mammary gland to carcinogenesis is influenced by its normal development, particularly during developmental stages such as puberty and pregnancy that are characterized by marked changes in proliferation and differentiation. Protein kinases are important regulators of proliferation and differentiation, as well as of neoplastic transformation, in a wide array of tissues, including the breast. Using a RT-PCR-based cloning strategy, we have identified 41 protein kinases that are expressed in breast cancer cell lines and in the murine mammary gland during development. The expression of each of these kinases was analyzed throughout postnatal mammary gland development as well as in a panel of mammary epithelial cell lines derived from distinct transgenic models of breast cancer. Although the majority of protein kinases isolated in this screen have no currently recognized role in mammary development, most kinases examined were found to exhibit developmental regulation. After kinases were clustered on the basis of similarities in their temporal expression profiles during mammary development, multiple distinct patterns of expression were observed. Analysis of these patterns revealed an ordered set of expression profiles in which successive waves of kinase expression occur during development. Interestingly, several protein kinases whose expression has previously been reported to be restricted to tissues other than the mammary gland were isolated in this screen and found to be expressed in the mammary gland. In aggregate, these findings suggest that the array of kinases participating in the regulation of normal mammary development is considerably broader than currently appreciated.

Key Words: **mammary gland; protein kinase; development; cell differentiation; carcinogenesis.**

INTRODUCTION

Numerous epidemiologic and animal studies analyzing the impact of reproductive events such as puberty, pregnancy, and parity on early events in carcinogenesis suggest that the developmental state of the breast plays a critical role in the determination of breast cancer risk (Lambe *et al.*, 1994; MacMahon *et al.*, 1970, 1982; Newcomb *et al.*, 1994; Russo and Russo, 1978, 1987). This implies an intrinsic relationship between the process of carcinogenesis and normal pathways of differentiation and development in the breast. Therefore, understanding the mechanisms by which reproductive events influence breast cancer susceptibility will undoubtedly require an improved understanding of normal mammary development, particularly with respect to genes that control mammary proliferation and differentiation.

Protein kinases represent the largest class of genes known to regulate differentiation, development, and carcinogenesis in eukaryotes. Therefore, we have chosen to study members of this family of regulatory proteins as one approach to elucidating the relationship between development and carcinogenesis in the breast. Several protein kinases have been implicated in the development of breast cancer either in humans or in rodent model systems. For instance, the EGF receptor and *ErbB2/HER2* are each amplified and overexpressed in subsets of highly aggressive breast cancers, and these molecules may thereby provide prognostic information relevant to clinical treatment and outcome (Klijn *et al.*, 1993; Slamon *et al.*, 1987, 1989). Furthermore, overexpression of specific protein kinases, or

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of ligands for protein kinases, in the mammary epithelium of transgenic animals results in neoplastic transformation (Cardiff and Muller, 1993; Guy *et al.*, 1994; Muller *et al.*, 1988, 1990). Finally, by analogy with hematopoiesis, some protein kinases are likely to be expressed in a lineage-restricted manner in the breast and as such may provide insight into biologically meaningful subpopulations of cells (Dymecki *et al.*, 1990; Siliciano *et al.*, 1992; Tsukada *et al.*, 1993). These findings suggest that further analysis of protein kinase function may reveal significant features of the relationship between development and carcinogenesis in the breast, as well as provide insight into how the decision to proliferate or differentiate is made in mammary epithelial cells.

In light of the importance of this class of regulatory molecules, we initiated a systematic study of the role of protein kinases in mammary gland development and carcinogenesis. Examination of 1450 cDNA clones generated using a RT-PCR-based screening strategy identified 41 protein kinases, including 33 tyrosine kinases and 8 serine/threonine kinases, 3 of which were novel. The expression of these kinases was subsequently examined during defined stages in mammary development and in a panel of mammary tumor cell lines derived from distinct transgenic models of breast cancer. Our findings reveal an ordered series of protein kinase expression patterns that occur during each of the stages of postnatal mammary development, suggesting that these molecules may be important regulators of this process.

MATERIALS AND METHODS

Cell Culture

Mammary epithelial cell lines were derived from mammary tumors or hyperplastic lesions that arose in MMTV-*c-myc*, MMTV-*int-2*, MMTV-*neu*/NT, or MMTV-Ha-*ras* transgenic mice and included: the *neu* transgene-initiated mammary tumor-derived cell lines SMF, NAF, NF639, NF11005, and NK-2; the c-*myc* transgene-initiated mammary tumor-derived cell lines 16MB9a, 8Mala, MBp6, M158, and M1011; the Ha-*ras* transgene-initiated mammary tumor-derived cell lines AC816, AC236, and AC711; the *int*-2 transgene-initiated hyperplastic cell line HBI2; and the *int*-2 transgene-initiated mammary tumor-derived cell line 1128 (Morrison and Leder, 1994). Additional cell lines were obtained from ATCC and included NIH3T3 cells and the nontransformed murine mammary epithelial cell lines NMuMG and CL-S1. All cells were cultured under identical conditions in DMEM medium supplemented with 10% bovine calf serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin.

Animals and Tissues

FVB mice were housed under barrier conditions with a 12-h light/dark cycle. The mammary glands from between 10 and 40 age-matched mice were pooled for each developmental point. Mice for pregnancy points were mated at 4–5 weeks of age. Mammary gland harvest consisted in all cases of the No. 3, 4, and 5 mammary glands. The lymph node embedded in the No. 4 mammary gland was removed prior to harvest. Tissues used for RNA preparation were snap frozen on dry ice. Tissues used for *in situ* hybridization analysis were embedded in OCT medium and frozen in a dry ice/isopentane bath. Developmental expression patterns for 13 kinases were confirmed using independent pools of RNA. Analysis of the developmental expression pattern for an additional kinase using these independent pooled samples revealed a similar pregnancy-upregulated expression pattern that differed with respect to the day of pregnancy at which maximal upregulation occurred.

Construction and Analysis of Kinase-Specific cDNA Libraries

Kinase-specific cDNA libraries were constructed using mRNA prepared from the mammary glands of mice at specified stages of development and from a panel of mammary epithelial cell lines. Specifically, total RNA was prepared from the mammary glands of either 5-week-old nulliparous female mice or parous mice that had undergone a single pregnancy followed by 21 days of lactation and 2 days of postlactational regression. Total RNA was also prepared from the mammary epithelial cell lines NMuMG, CL-S1, HBI2, SMF, 16MB9a, AC816, and 1128, described above.

First-strand cDNA was generated from each of these nine sources of RNA using the cDNA Cycle kit according to the manufacturer's directions (Invitrogen). These were amplified using degenerate oligonucleotide primers corresponding to conserved regions in kinase catalytic subdomains VI and IX. The degenerate primers, PTKIa (5′-GGGCCCGGATCCAC(A/C)G(A/G/C/T)GA-(C/T)(C/T)-3′) and PTKIIa (5′-CCCGGGGAATTCCA(A/T)AGG-ACCA(G/C)AC(G/A)TC-3′), have previously been shown to amplify a conserved 200-bp portion of the catalytic domain of a wide variety of tyrosine kinases (Wilks, 1989, 1991; Wilks *et al.*, 1989). Two additional degenerate oligonucleotide primers, BSTKIa (5′-GGGCCCGGATCC(G/A)T(A/G)CAC(A/C)G(A/G/C)GAC(C/T)T-3′) and BSTKIIa (5′-CCCGGGGAATTCC(A/G)(A/T)A(A/G)CTC-CA(G/C)ACATC-3′), that differed from PTKIa and PTKIIa were also designed for this study. Restriction sites, underlined in the primer sequences, were generated at the 5′ (*Apa*I and *Bam*HI) and 3′ (*Xma*I and *Eco*RI) ends of the primer sequences.

Each cDNA source was amplified in three separate PCR reactions using pairwise combinations of the PTKIa/PTKIIa, BSTKIa/BSTKIIa, or BSTKIa/PTKIIa degenerate primers. Following 5-min denaturation at 95°C, samples were annealed at 37°C for 1 min, polymerized at 63°C for 2 min, and denatured at 95°C for 30 s for 40 cycles. The resulting ~200-bp PCR products were purified from low-melting agarose (BMB), ligated into a T-vector (Invitrogen), and transformed in *Escherichia coli*. Following blue/white color selection, approximately 50 transformants were picked from each of the 27 PCR reactions (3 reactions for each of nine cDNA sources) and were subsequently transferred to gridded plates and replica plated. In total, 1450 transformants were analyzed.

Dideoxy sequencing of 100 independent transformants was performed, resulting in the identification of 14 previously described tyrosine kinases. In order to identify and eliminate additional isolates of these kinases from further consideration, filter lifts representing the 1350 remaining transformants were hybridized individually to radiolabeled DNA probes prepared from each of the 14 initially isolated kinases. Hybridization and washing were performed as described under final washing conditions of 0.1× SSC/0.1% SDS at 70°C that were demonstrated to prevent cross-hybridization between kinase cDNA inserts (Marquis *et al.*, 1995). In this manner, 887 transformants containing inserts from the 14 tyrosine kinases that had initially been isolated were identified.

Identifications made by colony hybridization were consistent with those made directly by DNA sequencing.

The remaining 463 transformants were screened by PCR using T7 and SP6 primers to identify those containing cDNA inserts of a length expected for protein kinases. One hundred seventy-two transformants were found to have cDNA inserts between 150 and 300 bp in length and were subjected to further analysis by successive rounds of dideoxy sequencing and colony lift hybridization. This resulted in the identification of 27 additional protein kinases.

Individual clones were sequenced using the Sequenase version 2 dideoxy chain termination kit (U.S. Biochemical Corp.). Putative protein kinases were identified by the DFG consensus located in catalytic subdomain VI. DNA sequence analysis was performed using MacVector 3.5 and the NCBI BLAST server.

RNA Preparation and Analysis

RNA was prepared by homogenization of snap-frozen tissue samples or tissue culture cells in guanidinium isothiocyanate supplemented with 7 μl/ml 2-mercaptoethanol followed by ultracentrifugation through cesium chloride as previously described (Marquis *et al.*, 1995; Rajan *et al.*, 1997). Poly(A)$^+$ RNA was selected using oligo(dT) cellulose (Pharmacia), separated on a 1.0% LE agarose gel, and passively transferred to a Gene Screen membrane (NEN). Northern hybridization was performed as described using ^{32}P-labeled cDNA probes corresponding to catalytic subdomains VI–IX of each protein kinase that were generated by PCR amplification of cloned catalytic domain fragments (Marquis *et al.*, 1995). In all cases calculated transcript sizes were consistent with values reported in the literature.

In Situ Hybridization

In situ hybridization was performed as described (Marquis *et al.*, 1995). Antisense and sense probes were synthesized with the Promega *in vitro* transcription system using ^{35}S-UTP and ^{35}S-CTP from the T7 and SP6 RNA polymerase promoters of a PCR template containing the sequences used for Northern hybridization analysis.

RESULTS

Identification of Protein Kinases Expressed in the Murine Mammary Gland

As an initial step in studying the role of protein kinases in regulating mammary proliferation and differentiation, we designed a screen to identify protein kinases expressed in the mammary gland and in breast cancer cell lines. An RT-PCR cloning strategy was employed that relies on the use of degenerate oligonucleotide primers corresponding to conserved amino acid motifs present within the catalytic domain of protein tyrosine kinases (Wilks, 1989; Wilks *et al.*, 1989). RNA prepared from nine different sources was used as starting material for the generation of kinase-specific cDNA libraries. These sources included mammary glands from 5-week-old nulliparous mice undergoing puberty and parous mice at day 2 of involution, as well as a panel of seven murine mammary epithelial cell lines. Cell lines consisted of two nontransformed mammary epithelial cell lines in addition to five cell lines derived from tumors and hyperplasias that arose in the mammary glands of MMTV-*neu*, MMTV-*c-myc*, MMTV-Ha-*ras*, or MMTV-*int2* transgenic mice (Leder *et al.*, 1986; Muller *et al.*, 1988, 1990; Sinn *et al.*, 1987). Mammary tumors arising in each of these transgenic strains have previously been demonstrated to possess distinct and characteristic histopathologies that have been described as a large basophilic cell adenocarcinoma associated with the *myc* transgene, a small eosinophilic cell papillary carcinoma associated with the Ha-*ras* transgene, a pale intermediate cell nodular carcinoma associated with the *neu* transgene, and a papillary adenocarcinoma associated with the *int*-2 transgene (Cardiff and Muller, 1993; Cardiff *et al.*, 1991; Munn *et al.*, 1995).

First-strand cDNA prepared from each of these sources was independently amplified using the previously described degenerate oligonucleotide primers, PTKI and PTKII, that encode conserved amino acid motifs within catalytic subdomains VIb and IX (Hanks and Quinn, 1991; Wilks, 1991; Wilks *et al.*, 1989). In an effort to isolate a broad array of protein kinases, two additional degenerate oligonucleotide primers, BSTKI and BSTKII, which are also directed against subdomains VIb and IX, but which differ in nucleotide sequence, were designed for use in this screen. Degenerate oligonucleotide primers were used in three pairwise combinations (PTKI/PTKII, BSTKI/BSTKII, and BSTKI/PTKII) to amplify first-strand cDNA from each of the nine sources. The resulting 150- to 300-bp PCR products from each amplification were subcloned into a plasmid vector. Approximately 50 bacterial transformants from each of the 27 PCR reactions were replica plated and screened by a combination of DNA sequencing and colony lift hybridization in order to identify the protein kinase from which each subcloned catalytic domain fragment was derived.

A total of 1450 bacterial transformants were analyzed by this approach. Of these, greater than 70% contained protein kinase cDNA inserts as determined by hybridization and sequencing. Analysis of these clones resulted in the identification of 33 tyrosine kinases and 8 serine/threonine kinases (Table 1). The 19 receptor tyrosine kinases and 14 cytoplasmic tyrosine kinases isolated accounted for all but 33 of the 1056 kinase-containing clones. The remaining clones were derived from 8 serine/threonine kinases, 7 of which were represented by a single clone each, including each of the novel kinases isolated in this screen. Approximately half of the 41 kinases were isolated more than once, and most of these were isolated from more than one tissue or cell line (Table 1 and data not shown). Eight tyrosine kinases, including *Jak2, Fgfr1, EphA2, Met, Igf1r, Hck, Jak1,* and *Neu,* accounted for 830 (79%) of all clones analyzed (Table 1). Conversely, 18 kinases (44%) were represented by a single clone each, suggesting that further screening of cDNA libraries derived from these tissues and cell lines may yield additional kinases. The number of clones isolated for each kinase presumably reflects a combination of mRNA abundance and extent of homology to the oligonucleotide primers used in the amplification reaction.

Three novel protein kinases were identified in this

TABLE 1
Protein Kinases Isolated from Mammary Glands and Mammary Epithelial Cell Lines

Receptor tyrosine kinases		Nonreceptor tyrosine kinases		Serine/threonine kinases	
Axl/Ufo	6	c-Abl	5	c-Akt1	1
EphA2	121	Csk	46	Mlk1	1
EphA7	1	Ctk	1	Plk	26
EphB3	2	c-Fes	24	A-Raf	1
Egfr	1	Fyn	7	SLK	1
Fgfr1	126	Hck	88		
Flt3	1	Jak1	74		
Igf1r	89	Jak2	150		
InsR	1	Lyn	21		
c-Kit	2	Prkmk3	3	Novel kinases	
Met	120	c-Src	23		
MuSK	1	Srm	1	Bstk1	1
Neu	62	Tec	1	Bstk2	1
Ron	10	Tyk2	4	Bstk3	1
Ryk	1				
Tie1	1				
Tie2	27				
Tyro10	2				
Tyro3	1				

Note. Kinases are arranged by family and class. The number of clones isolated for each kinase is shown on the right.

screen, designated *Bstk1, 2,* and *3*. Each of these kinases contains the amino acid motifs characteristic of serine/threonine kinases (Fig. 1). *Bstk1* was isolated from a mammary epithelial cell line derived from a tumor that arose in an MMTV-*neu* transgenic mouse and is most closely related to the *SNF1* family of serine/threonine kinases. A full-length cDNA encoding *Bstk1* has subsequently been isolated (Gardner *et al.*, 2000b), as have partial cDNA sequences (Korobko *et al.*, 1997). *Bstk2* and *Bstk3* were each isolated from the mammary glands of mice undergoing early postlactational regression. *Bstk2* exhibits highest homology to kinases recently identified in *Saccharomyces cerevisiae* and *Arabidopsis thaliana* and analysis of full-length clones encoding this kinase indicates that it represents the first vertebrate member of a new family of mammalian protein kinases (Kurioka *et al.*, 1998; Ligos *et al.*, 1998; Stairs *et al.*, 1998). *Bstk3* is most closely related to calcium/calmodulin-dependent protein kinase I, and full-length isoforms have subsequently been identified in the mouse and rat (Yokokura *et al.*, 1997, and Gardner *et al.*, 2000a).

Expression of Protein Kinases in Mammary Epithelial Cell Lines

As a first step in investigating the range of expression patterns for the 41 protein kinases isolated in this screen, we determined kinase expression profiles in a panel of 18 murine mammary epithelial cell lines (Fig. 2). These included 14 cell lines derived from independent tumors arising in transgenic mice expressing either the *neu, c-myc,* H-*ras,* or *int-2* oncogenes under the control of the MMTV LTR, 1 cell line derived from the hyperplastic mammary epithelium of an MMTV-*int*-2 transgenic mouse, and 3 nontransformed, nontransgenic mammary epithelial cell lines (Fig. 2) (Leder *et al.*, 1986; Morrison and Leder, 1994; Muller *et al.*, 1988, 1990; Sinn *et al.*, 1987). Kinase expression was also investigated in NIH3T3 fibroblasts in order to identify those kinases that might be expressed in a mesenchymal- or epithelial-specific manner. All cell lines were grown under identical conditions and were harvested while actively proliferating.

Of the 41 kinases isolated in this screen, 25 were found to be ubiquitously expressed in the epithelial cell lines examined (Fig. 2 and data not shown). Steady-state mRNA levels for 11 of these ubiquitously expressed kinases exhibited relatively little variation among cell lines. These include *Tyk2, Neu, Ryk, Plk, Csk, Akt1, A-Raf, Prkmk3,* and the insulin receptor. Steady-state mRNA levels for the remaining 14 ubiquitously expressed kinases varied considerably among cell lines. These include the receptor tyrosine kinases *Egfr, Igf1R, Met, Tyro3, EphA2, EphA7/Hek11/Ehk3, and EphB3/Hek2,* as well as the cytoplasmic tyrosine kinases *Jak1, Jak2, c-Abl, c-Src, Lyn, and Tec* and the serine/threonine kinase *SLK* (Fig. 2 and data not shown).

In contrast, mRNA expression of 11 of the kinases examined was detectable in only a subset of epithelial cell lines. These kinases range from those that are expressed within the majority of cell lines tested, such as *Fgfr1, Fyn, Axl,* and *Mlk1,* to kinases that are expressed in only a small number of these cell lines, such as *Tyro10, c-Fes, c-Kit,* and *Flt3* (Fig. 2 and data not shown). Within this latter group of kinases, *Ron, Srm,* and *Hck* are expressed at detectable

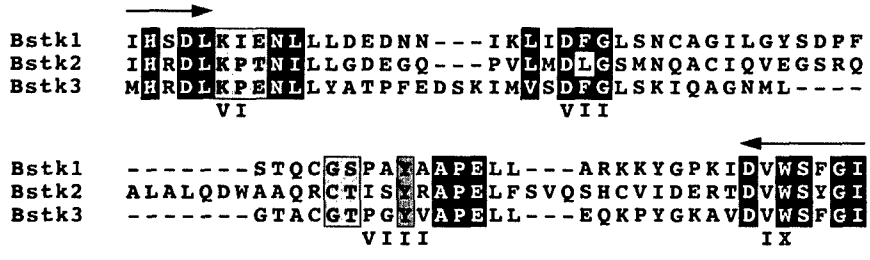

FIG. 1. Amino acid sequence of catalytic subdomains of novel protein kinases. Aligned amino acid sequences for isolated cDNA fragments corresponding to catalytic subdomains VIb–IX of *Bstk1, Bstk2,* and *Bstk3.*

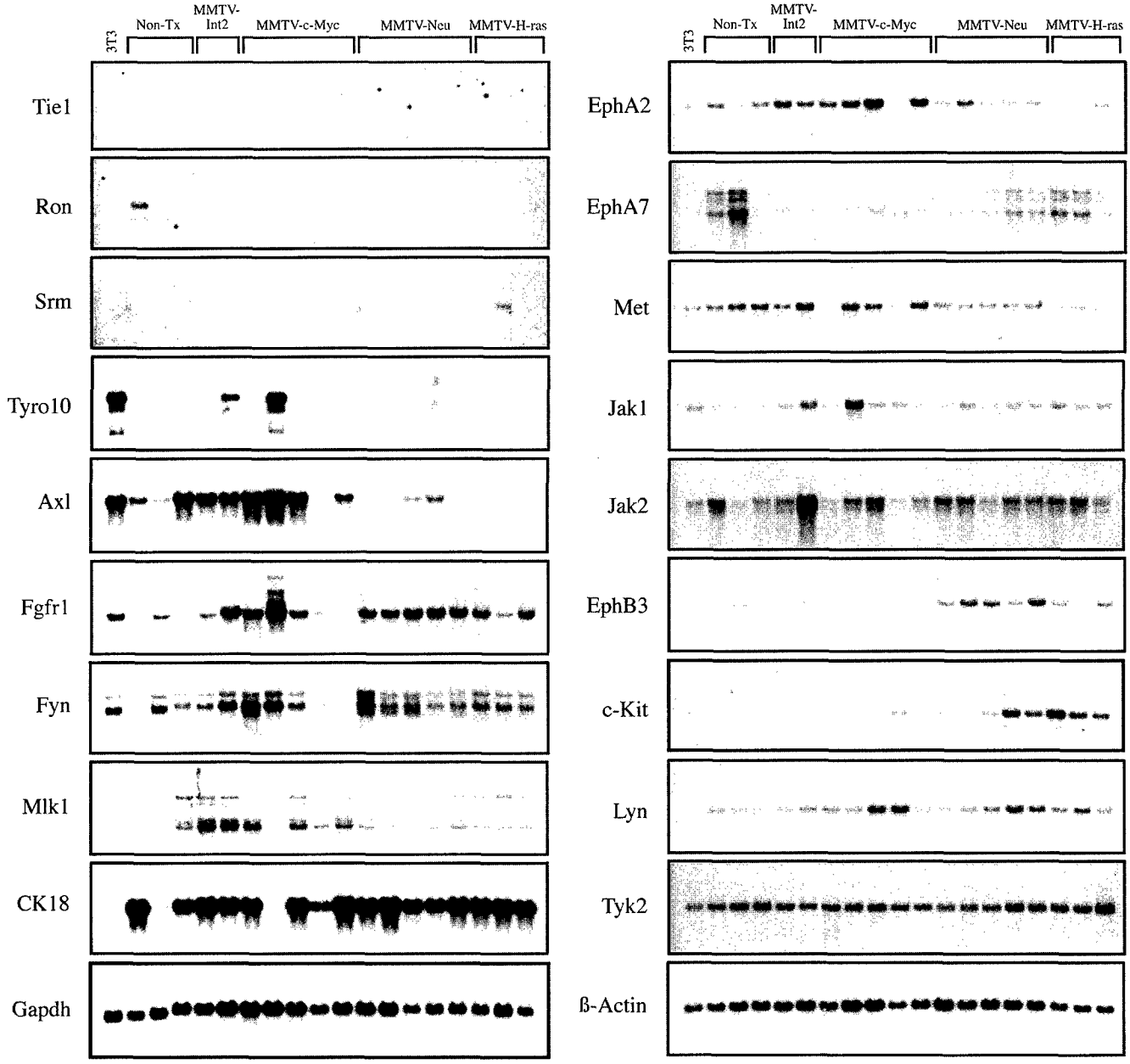

FIG. 2. Protein kinase expression in murine breast cancer cell lines. Transformed cell lines were derived from mammary adenocarcinomas or mammary hyperplasias (HBI2) arising in transgenic animals expressing the *int-2*, c-*myc*, *neu*, or v-Ha-*ras* oncogenes in the mammary gland, as indicated. Northern hybridization analysis of 6 μg of poly(A)$^+$ RNA from actively growing murine cell lines hybridized with cDNA probes specific for each of the kinases indicated is shown. Origins of the cell lines from left to right are as follows: NIH3T3 fibroblast, nontransformed (Non-Tx) (NMuMG, HC11, and CL-S1), MMTV-*int-2* (HBI2 and 1128), MMTV-c-*myc* (8Ma1a, MBp6, M1011, M158, and 16MB9a), MMTV-*neu* (SMF, NAF, NF639, NF11005, and NK-2), and MMTV-Ha-*ras* (AC816, AC711, and AC236).

levels in only a single mammary epithelial cell line each (Fig. 2 and data not shown). Interestingly, both *EphB3* and *c-Kit* are preferentially expressed in tumor cell lines derived from MMTV-*neu* and MMTV-Ha-*ras* transgenic animals compared to cell lines derived from MMTV-c-*myc* and MMTV-*int-2* transgenic animals. Oncogene-associated patterns of expression have also been observed for *Bstk1* and *Bstk3* (Gardner *et al.*, unpublished results). Similar patterns of expression in this panel of cell lines have previously been reported for protein tyrosine phosphatase ϵ and other molecules (Elson and Leder, 1995; Morrison and Leder, 1994).

Additional patterns of expression that presumably reflect cell-type specificity were observed. For instance, *Mlk1* is expressed in all cell lines except for NIH3T3 fibroblasts and

for MBp6, the sole mammary cell line that does not express the epithelial marker cytokeratin 18. The resulting inference that *Mlk1* expression is epithelial-specific was subsequently confirmed by *in situ* hybridization (Fig. 8). By comparison with *Mlk1*, *Tyro10* exhibited an inverse pattern of expression with steady-state levels of mRNA detectable only in NIH3T3, MBp6, and the int-2-initiated tumor cell line, 1128, suggesting that this kinase is preferentially expressed in stromal compared to epithelial cells. This hypothesis was also confirmed by *in situ* hybridization (Figs. 10E–10H). Similarly, expression in mammary epithelial cell lines was detected neither for *Tie1* nor *Tie2*, each of which has been shown to be expressed in an endothelial-specific manner, nor for *MuSK*, whose expression is restricted to muscle (Ganju *et al.*, 1995; Partanen *et al.*, 1992; Sato *et al.*, 1993; Valenzuela *et al.*, 1995b).

Surprisingly, expression of the receptor tyrosine kinases *EphB3/Hek2* and *EphA7/Hek11/Ehk3* was demonstrated in all of the mammary epithelial cell lines examined, despite the fact that expression of these kinases has been reported to be restricted primarily to the central nervous system (Aasheim *et al.*, 1997; Adams *et al.*, 1999; Bergemann *et al.*, 1998; Fox *et al.*, 1995; Krull *et al.*, 1997; Valenzuela *et al.*, 1995a). Similarly, despite previous reports that expression of each of the nonreceptor tyrosine kinases *Lyn*, *Tec*, and *Hck* is restricted primarily to cells of hematopoietic origin, *Lyn* and *Tec* expression was detected in all 18 mammary epithelial cell lines tested, and *Hck* expression was detected in 2 mammary tumor cell lines (Fig. 2 and data not shown) (Kluppel *et al.*, 1997; Quintrell *et al.*, 1987; Sato *et al.*, 1994; Umemori *et al.*, 1992; Yi *et al.*, 1991; Ziegler *et al.*, 1987). Interestingly, *Lyn* has been shown to specifically bind and phosphorylate *Tec* in hematopoietic cells *in vivo*, suggesting that *Tec* is a downstream effector of *Lyn* (Mano *et al.*, 1994, 1996). Our finding that *Lyn* and *Tec* are also coexpressed in mammary epithelial cells suggests that this signaling pathway may function in mammary epithelial cells as well as in cells of hematopoietic origin.

Expression of Protein Kinases during Postnatal Mammary Development

Since the expression of regulatory molecules is frequently controlled at the level of transcription, we analyzed the temporal pattern of expression during postnatal mammary development for each of the protein kinases isolated in this screen. Kinase expression was determined in mammary glands harvested from male FVB mice and from female mice at nine time points corresponding to developmental milestones encompassing puberty (2, 5, and 10 weeks of age), pregnancy (days 7, 14, and 20), lactation (day 9), and postlactational regression (days 2 and 7). Replicate Northern blots containing poly(A)$^+$ mRNA from each of these developmental stages were hybridized with probes prepared from catalytic domain fragments corresponding to each kinase.

As an initial control, Northern blots were hybridized with probes for the genes encoding β-actin, Gapdh, and cytokeratin 18 (Fig. 3). The resulting patterns were consistent with previous observations that steady-state levels of mRNA for many genes appear to decline during lactation and, to a lesser extent, late pregnancy and early postlactational regression (Marquis *et al.*, 1995; Rajan *et al.*, 1997). Since the expression of *β-actin*, *gapdh*, and *cytokeratin 18* does not decrease on a per-cell basis when assayed by *in situ* hybridization (J. Hartman, unpublished results), this phenomenon most likely results from a dilutional effect due to the extraordinary increase in milk protein gene expression that occurs during lactation. Expression levels for each kinase were therefore quantitated by phosphorimager analysis and normalized to *β-actin* in order to correct for these dilutional effects.

A wide range of developmental patterns of gene expression was observed for the kinases surveyed in this study.

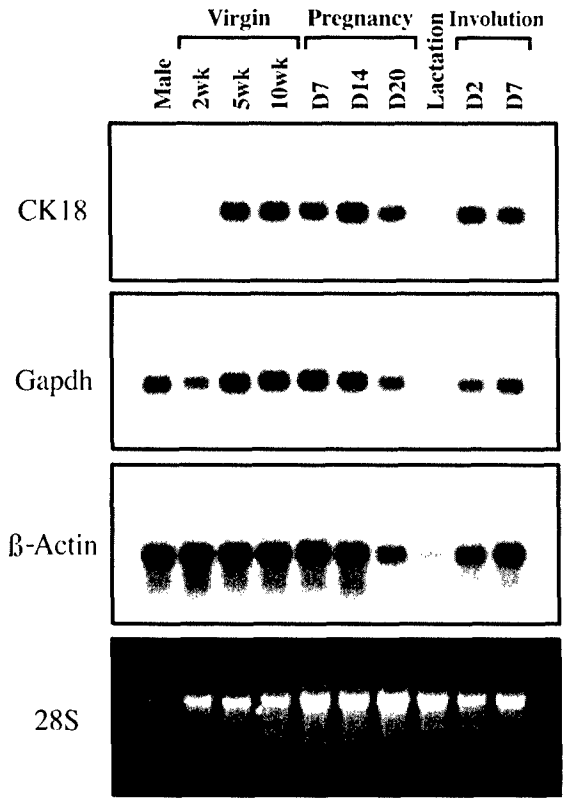

FIG. 3. Expression of control genes during mammary gland development. Northern hybridization analysis of mRNA expression is shown for cytokeratin 18, Gapdh, and β-actin, during postnatal developmental of the murine mammary gland. 3 μg of poly(A)$^+$ RNA isolated from the mammary glands of FVB mice at the indicated time points was hybridized to ^{32}P-labeled cDNA probes for the genes indicated. The smear of poly(A)$^+$ RNA beneath the 28S ribosomal RNA band is shown as a loading control. Note the apparent decrease in gene expression levels during lactation for all three genes, despite the similar amount of poly(A)$^+$ present. Origins of the mammary developmental time points are as follows: adult male; nulliparous females at 2 weeks (prior to puberty), 5 weeks (during puberty), and 10 weeks (following puberty) of age; gravid females at day 7, day 14, and day 20 of pregnancy; day 9 of lactation; and day 2 and day 7 of postlactational regression.

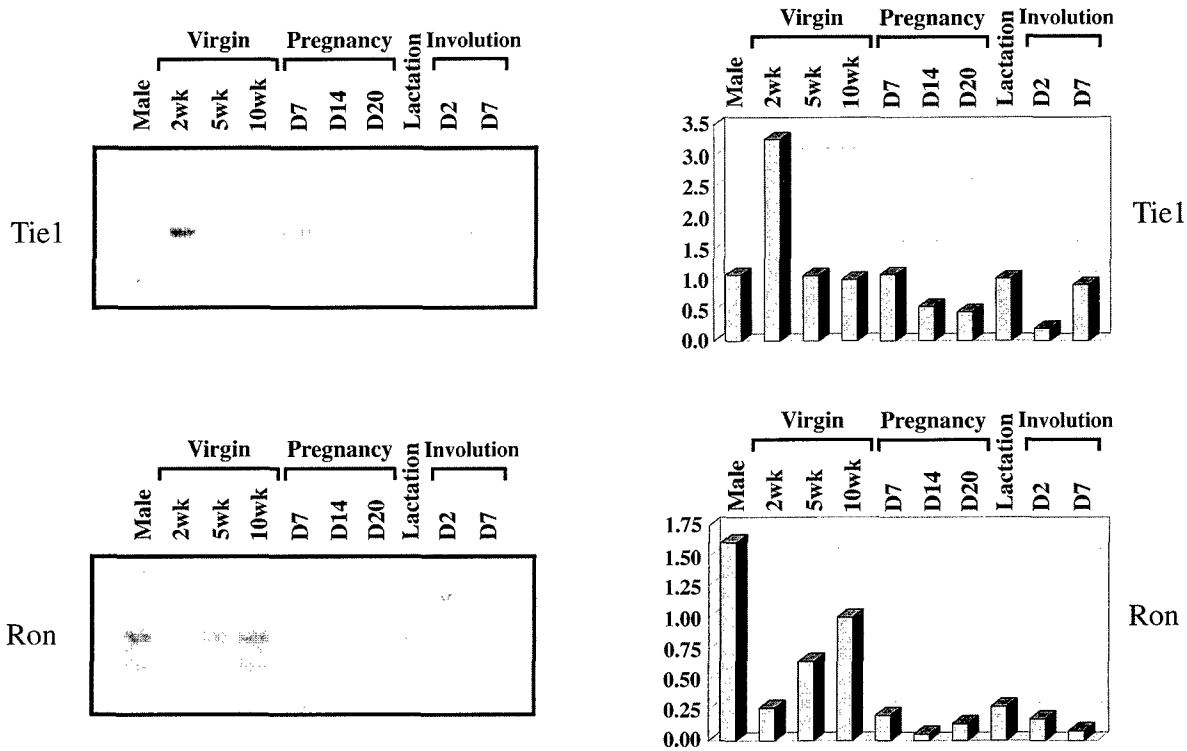

FIG. 4. Expression of protein kinases during ductal morphogenesis. Northern hybridization analysis of *Tie1* and *Ron* expression during postnatal development of the murine mammary gland is shown. 3 μg of poly(A)$^+$ RNA isolated from the mammary glands of FVB mice at the indicated time points was hybridized to ^{32}P-labeled cDNA probes for the genes indicated. Origins of the mammary developmental time points are as in Fig. 3. Phosphorimager analysis of Northern blots is shown for *Tie1* and *Ron*. Protein kinase expression was quantitated and normalized to *β-actin* expression to correct for dilutional effects due to large-scale increases in milk protein gene expression during late pregnancy and lactation. Expression levels normalized to β-actin are shown relative to adult virgin (10 wk).

Based on the assumption that kinases exhibiting distinctive patterns of regulation during particular stages of mammary development may be involved in regulating specific developmental events, we grouped these genes according to similarities in their developmental expression profiles. This approach revealed an ordered set of expression profiles that suggested the coordinated regulation of protein kinases acting at different stages of mammary development. While the observation that a particular kinase is expressed in a developmentally regulated manner does not prove that the kinase plays a role in development, the spatial and temporal patterns of expression for a gene often provide important clues to its biological function. Similarly, the identification of kinases with common developmental expression profiles may identify kinases whose developmental functions are related.

Kinase Expression during Ductal Morphogenesis

Prior to the onset of puberty at 2 weeks of age, the mammary glands of female FVB mice consist of a rudimentary epithelial tree originating from the nipple and penetrating a short distance into a mammary fat pad composed of fibroblasts and adipocytes. Following the onset of puberty at approximately 3 weeks of age, increased levels of ovarian steroids trigger the formation of club-shaped terminal end buds at the ends of growing mammary ducts. These are composed of highly proliferative, relatively undifferentiated epithelial cells that give rise to the differentiated cell types of the mammary tree, and the appearance of these structures marks the onset of the rapid cellular proliferation characteristic of ductal morphogenesis. By 5 weeks of age, over half of the mammary fat pad is filled with epithelial ducts as a consequence of ongoing ductal elongation and branching. The completion of ductal morphogenesis occurs at 10 weeks of age when most terminal end buds have reached the edge of the fat pad and have regressed.

Unlike many strains of mice in which males lack mammary glands, mammary glands are present in male FVB mice and can be studied. This represents one advantage of studying mammary development in this strain of mice. Accordingly, several protein kinases, including c-*Abl*, *Met*, *Csk*, *hck*, c-*Src*, *Fgfr1*, *Axl*, *Jak1*, *Jak2*, *Tyro3*, *Mlk1*, and *Ctk*, exhibited similar levels of expression in the mammary glands of adult male mice and 2-week-old female mice (Figs. 4–9). Presumably, this reflects the fact that both adult males and prepubescent females have low levels of circulating 17β-estradiol and possess only a rudimentary mammary epithelial tree. Conversely, levels of expression for

Tie1, *Ron*, *SLK*, and *Plk* differed in the mammary glands of adult male and 2-week-old female mice, potentially reflecting different levels of androgens, different diets (e.g. chow versus milk), or other age-specific or gender-specific differences between these two groups of mice. Several protein kinases, including *Ron*, *Met*, c-*Abl*, *Axl*, *Jak1*, *Tyro3*, and *Mlk1*, exhibited an increase in expression in the mammary glands of nulliparous mice between 2 and 5 weeks of age concomitant with the onset of ductal morphogenesis (Figs. 4–7). This pattern may reflect increases in circulating steroid hormone levels, increases in cellular proliferation, or increases in epithelial cell content (as reflected by the expression pattern of cytokeratin 18), that occur at the onset of puberty or changes in diet that occur at weaning (Fig. 3). Thus, similarities and differences in diet, hormonal environment, cellular proliferation, cellular differentiation, and epithelial content may account for changes in kinase expression patterns observed at different developmental stages.

The tyrosine kinases *Tie1*, *Ron*, and *Srm* each exhibited unique expression patterns with highest steady-state levels of mRNA occurring during the development of the virgin gland (Fig. 4 and data not shown). The endothelial-specific receptor tyrosine kinases *Tie1* and *Tie2* were each found to be expressed at highest levels just prior to the onset of puberty in 2-week-old female mice (Fig. 4 and data not shown). A similar pattern of developmental expression was observed for the nonreceptor tyrosine kinase, *Srm* (data not shown). Little is currently known regarding the physiological state of the prepubescent mammary gland. However, since *Tie1* and *Tie2* are required for the normal growth and organization of blood vessels, as well as for establishing the structural integrity of the vascular endothelium (Sato *et al.*, 1995), this observation suggests the possibility that changes in endothelial cells or in the vasculature of the mammary gland may precede the rapid ductal growth that begins at puberty.

In contrast to *Tie1* and *Tie2*, expression of the receptor tyrosine kinase *Ron* increases progressively during ductal morphogenesis, is downregulated at the onset of pregnancy, and remains low throughout the remainder of postnatal mammary development (Fig. 4). Since the ligand for *Ron*, Macrophage-Stimulating Protein, is a motility factor that promotes integrin-dependent epithelial cell migration (Wang *et al.*, 1996), it is plausible to hypothesize that *Ron* may contribute to the rapid epithelial migration characteristic of ductal morphogenesis. Consistent with this hypothesis, activation of *Ron* in epithelial cell lines results in enhanced proliferation, migration, and invasion through reconstituted basement membranes (Santoro *et al.*, 1996; Tamagnone and Comoglio, 1997; Wang *et al.*, 1996). Moreover, *Ron* is overexpressed in a subset of human primary breast carcinomas (Maggiora *et al.*, 1998). Together, these observations suggest a potential role for *Ron* in epithelial invasion during both normal and neoplastic mammary development.

Kinase Expression during Pregnancy and Lactation

Early in pregnancy, alveolar epithelial cells proliferate rapidly to form alveolar buds in response to rising levels of estrogens and progesterone. Alveolar cell proliferation occurs primarily during the first half of pregnancy, whereas alveolar differentiation occurs in a graded and progressive manner throughout pregnancy. This culminates in the withdrawal of epithelial cells from the cell cycle late in pregnancy concomitant with their terminal differentiation. Lactation, the final stage of lobuloalveolar development, occurs following parturition in the hormonal setting of high prolactin levels and declining estrogen and progesterone levels. The marked cellular changes that occur in the mammary gland during pregnancy and lactation are reflected on a molecular level by the temporally ordered expression of different milk protein genes (Robinson *et al.*, 1995). Each of the members of this class of genes undergoes a maximal increase in expression at a characteristic time during pregnancy and can be classified as an early, intermediate, or late marker for mammary epithelial differentiation.

Similar to milk protein genes, clustering of protein kinases on the basis of similarities in their developmental expression patterns also yields an ordered temporal set of expression profiles throughout pregnancy and lactation (Figs. 5 and 6). Consistent with the dramatic changes that take place in the mammary gland during these developmental stages, over half of all kinases examined were regulated during lobuloalveolar development. These were grouped into two sets based on whether kinases were upregulated or downregulated during pregnancy.

Seventeen kinases were found to be upregulated during pregnancy, and examination of their temporal expression profiles indicates that successive waves of kinase expression occur at each stage of lobuloalveolar development (Fig. 5). Ten kinases, including *EphA7*, *SLK*, c-*Abl*, *Met*, *Lyn*, c-*Kit*, and *Egfr*, exhibited maximal upregulation during early pregnancy. A smaller number of kinases exhibited maximal upregulation during the remainder of lobuloalveolar development, including mid-pregnancy (*Hck*) as well as late pregnancy and lactation (c-*Akt1* and c-*Fes*).

Expression of the receptor tyrosine kinase *EphA7* in tissues of adult mice has principally been described in the central nervous system. In this study, we detected *EphA7* expression both in mammary epithelial cell lines and in the mammary gland, where it is maximally upregulated during early pregnancy. Interestingly, during fetal development *EphA7* is expressed in bone marrow pro-B and pre-B cells, but not in more mature fetal B-lineage cells or in any B-lineage cells of the adult (Aasheim *et al.*, 1997). In light of the similarities between postnatal mammary development and the embryonic development of other organs, it is possible that *EphA7* may play a lineage-specific or differentiation-dependent role in the mammary gland during early pregnancy.

The receptor tyrosine kinase *Met* has previously been implicated in mammary development by virtue of its ability to stimulate branching morphogenesis and lumen for-

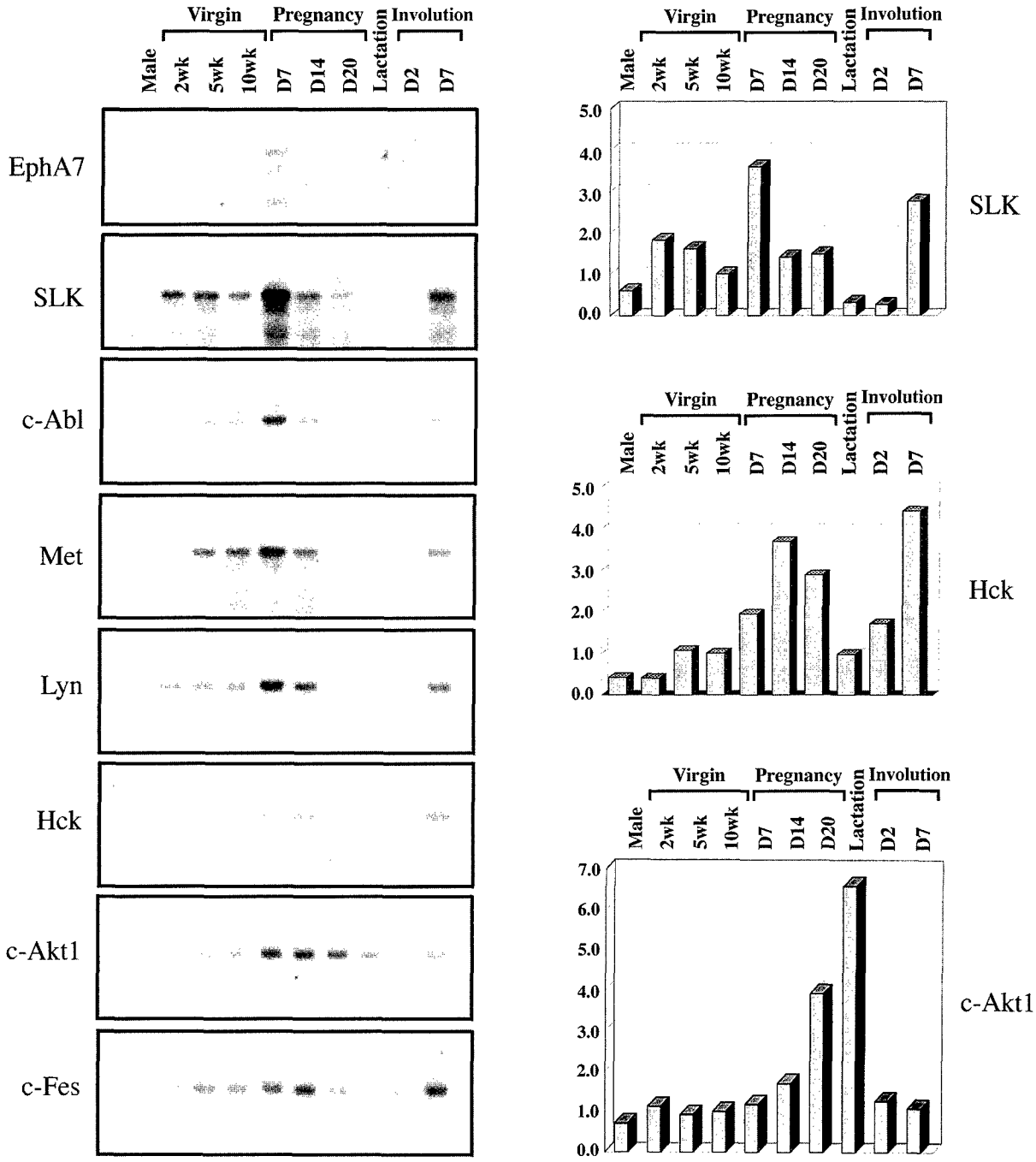

FIG. 5. Expression of protein kinases upregulated during pregnancy. Northern hybridization analysis of protein kinase expression during postnatal development of the murine mammary gland is shown for a selection of kinases that are upregulated during pregnancy. 3 μg of poly(A)$^+$ RNA isolated from the mammary glands of FVB mice at the indicated time points was hybridized to ^{32}P-labeled cDNA probes for the genes indicated. Northern blots are arranged with kinases exhibiting upregulation early in pregnancy at the top and kinases exhibiting upregulation late in pregnancy at the bottom. Origins of the mammary developmental time points are as in Fig. 3. Phosphorimager analyses of selected Northern blots are shown on the right. Expression levels normalized to β-actin are shown relative to adult virgin (10 wk).

mation in mammary epithelial cells (Niemann *et al.*, 1998; Tsarfaty *et al.*, 1992). In addition, some studies have suggested that c-*Met* is overexpressed in a subset of human breast cancers, and the mammary glands of transgenic mice expressing the *tpr-met* oncogene develop hyperplastic alveolar nodules and carcinomas (Jin *et al.*, 1997; Liang *et al.*, 1996). Nevertheless, a role for Met in mammary development has not been directly demonstrated.

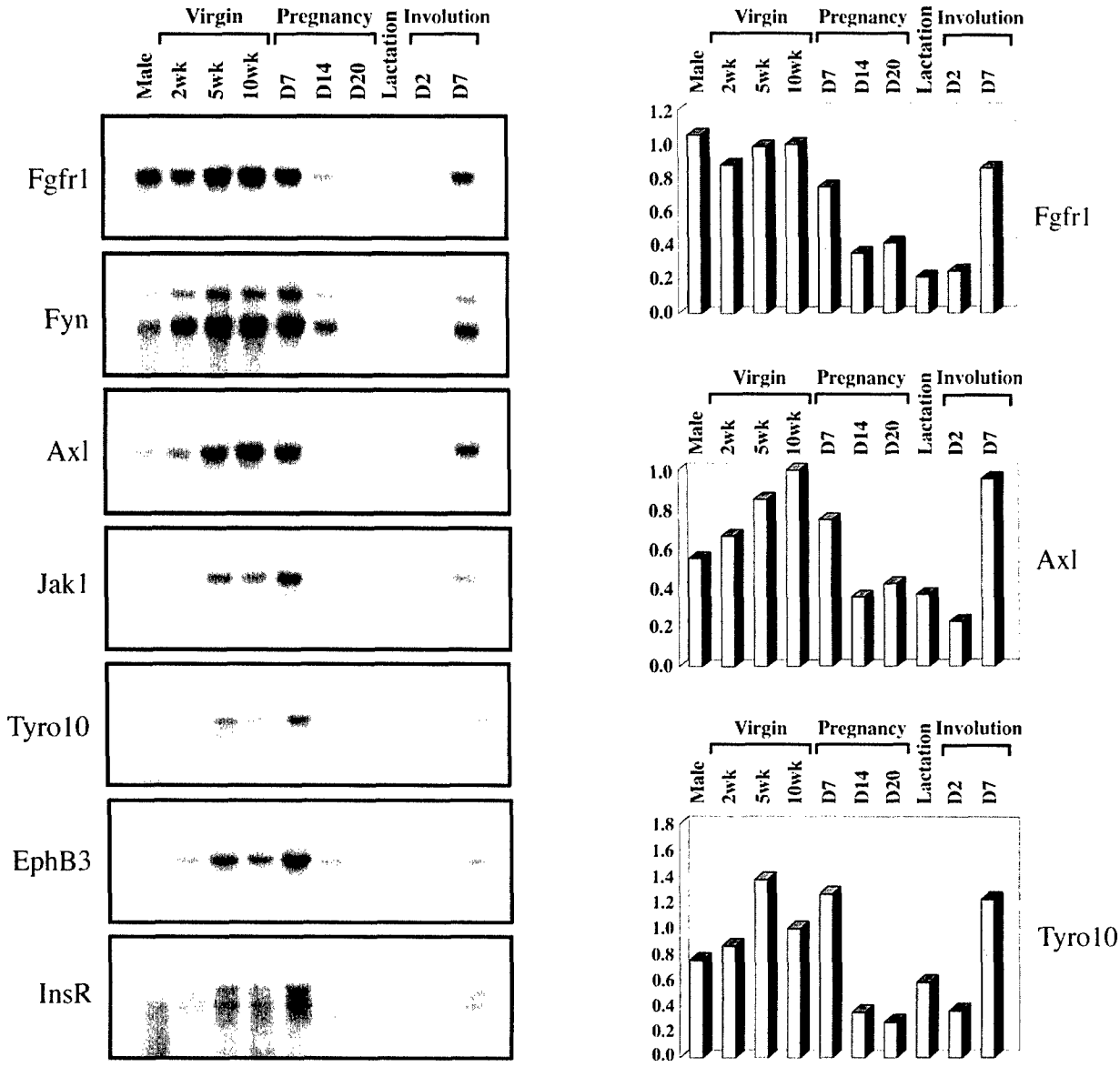

FIG. 6. Expression of protein kinases downregulated during pregnancy. Northern hybridization analysis of protein kinase expression during postnatal development of the murine mammary gland is shown for a selection of kinases that are downregulated during pregnancy. 3 μg of poly(A)$^+$ RNA isolated from the mammary glands of FVB mice at the indicated time points was hybridized to ^{32}P-labeled cDNA probes for the genes indicated. Origins of the mammary developmental time points are as in Fig. 3. Phosphorimager analyses of selected Northern blots are shown on the right. Expression levels normalized to β-actin are shown relative to adult virgin (10 wk).

Unlike other kinases analyzed in this study, both c-*Akt1* and c-*Fes* were maximally upregulated in the mammary gland during lactation. Akt1 has recently been shown to provide survival signals in response to a variety of growth factors and cytokines, and the Akt pathway is suppressed by the *PTEN* tumor suppressor gene. The further finding that germ-line mutations in *PTEN* predispose women to breast cancer suggests that Akt1 may be a prosurvival signal in the mammary epithelium as well (Li *et al.*, 1997, 1998; Liaw *et al.*, 1997; Stambolic *et al.*, 1998; Steck *et al.*, 1997). Consistent with this hypothesis, *Akt1* is expressed at high levels in human breast cancer cell lines. Similarly, while c-*Fes* expression has not previously been reported in the mammary gland, this kinase has been implicated in the induction of terminal myeloid differentiation and in promoting the survival of differentiating myeloid cells (Ferrari *et al.*, 1994; Manfredini *et al.*, 1997; Yu *et al.*, 1989). The high levels of *Akt1* and c-*Fes* expression observed in the mammary gland during lactation suggest that these kinases may play a role in propagating survival signals in terminally differentiated cells. As such, the rapid downregulation of *Akt1* and c-*Fes* expression at parturition may contribute to

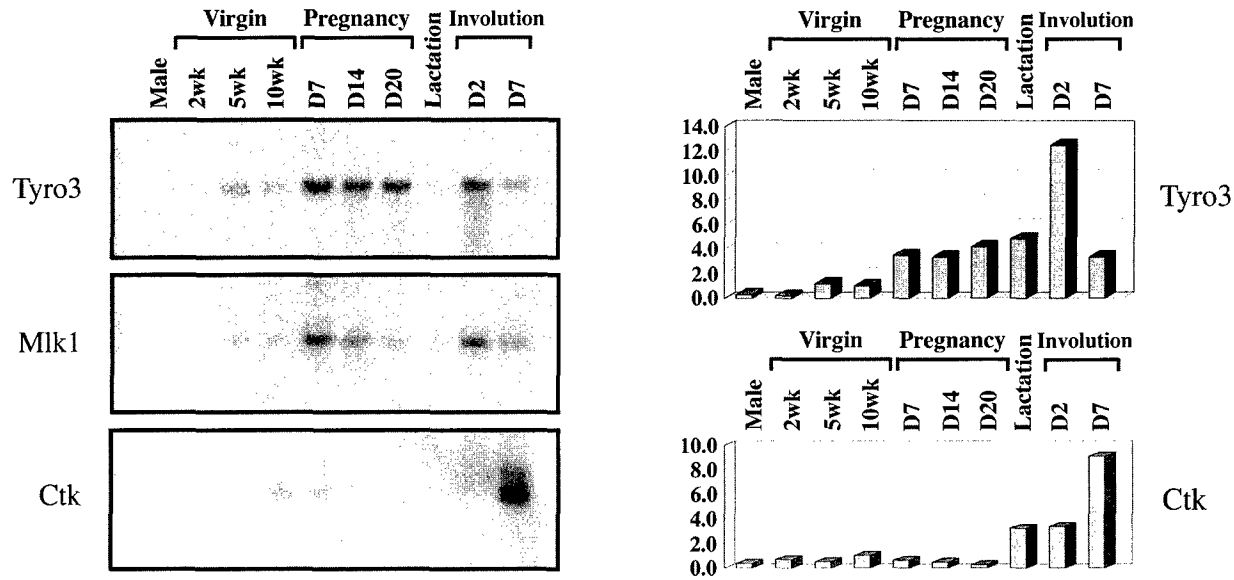

FIG. 7. Expression of protein kinases during postlactational involution. Northern hybridization analysis of protein kinase expression during postnatal development of the murine mammary gland is shown for protein kinases that are upregulated during involution. 3 μg of poly(A)$^+$ RNA isolated from the mammary glands of FVB mice at the indicated time points was hybridized to ^{32}P-labeled cDNA probes for the genes indicated. Origins of the mammary developmental time points are as in Fig. 3. Phosphorimager analyses of Northern blots for *Tyro3* and *Ctk* are shown on the right. Expression levels normalized to β-actin are shown relative to adult virgin (10 wk).

the onset of large-scale apoptosis at day 2 of postlactational involution. Such a model is consistent with the hypothesis that terminally differentiated cells are dependent on survival signals from hormones and growth factors to prevent death (Wyllie *et al.*, 1992).

Ten protein kinases were found to be downregulated in the mammary gland during pregnancy. In most cases, no marked changes in expression were observed until day 14 of pregnancy. However, for each kinase downregulation persisted throughout late pregnancy, lactation, and early postlactational regression (Fig. 6). The function of most of the downregulated kinases in mammary development is unknown. The majority of downregulated tyrosine kinases are growth factor receptors. These include *Fgfr1, Axl,* and the insulin receptor, each of which appears to mediate mitogenic responses in mammary epithelial cells. Overexpression of *Fgfr1* and of the insulin receptor has been described in subsets of human breast cancers (Adnane *et al.*, 1991; Ugolini *et al.*, 1999; Webster *et al.*, 1996). Conversely, downregulation of mitogenic growth factor pathways during mid- and late pregnancy may be required for the withdrawal of epithelial cells from the cell cycle that accompanies terminal differentiation. This hypothesis is consistent with the finding that downregulation of Fgfr-mediated signaling is required for the terminal differentiation of myogenic cells during avian development (Itoh *et al.*, 1996).

Alternately, changes in the expression of some receptor tyrosine kinases, such as the insulin receptor, may reflect the marked metabolic changes that occur in mammary gland during pregnancy.

Kinase Expression during Postlactational Involution

Immediately following weaning, secretory alveoli rapidly involute as the majority of mammary epithelial cells die in the apoptotic process of postlactational regression or involution. Cell death begins within 2 days following weaning, and by day 7 of postlactational involution, intensive remodeling of the mammary epithelium, stroma, and extracellular matrix is well underway.

Three protein kinases identified in this screen were found to be upregulated in the involuting mammary gland (Fig. 7). Expression of the receptor tyrosine kinase *Tyro3* is dramatically upregulated in the mammary gland at day 2 of involution, yet returns to preregression levels by day 7. Since *Tyro3* has been proposed to play a role in the growth and remodeling of the central nervous system, it is possible that it plays an analogous role in the mammary gland (Lai *et al.*, 1994; Stitt *et al.*, 1995).

Like *Tyro3, Mlk1* is also maximally upregulated in the mammary gland at day 2 of involution (Fig. 7). The developmental expression profiles of *Mlk1* and *Tyro3* share additional similarities as each kinase undergoes a modest initial upregulation in the mammary glands of nulliparous mice between 2 and 5 weeks of age and is further upregulated at the onset of pregnancy.

The developmental pattern of *Mlk1* expression was further investigated by *in situ* hybridization (Fig. 8). This revealed that *Mlk1* is expressed in the mammary gland in an epithelial-specific manner, as was predicted based on the similarity of its expression pattern in cell lines to that of cytokeratin 18. During puberty, *Mlk1* is preferentially ex-

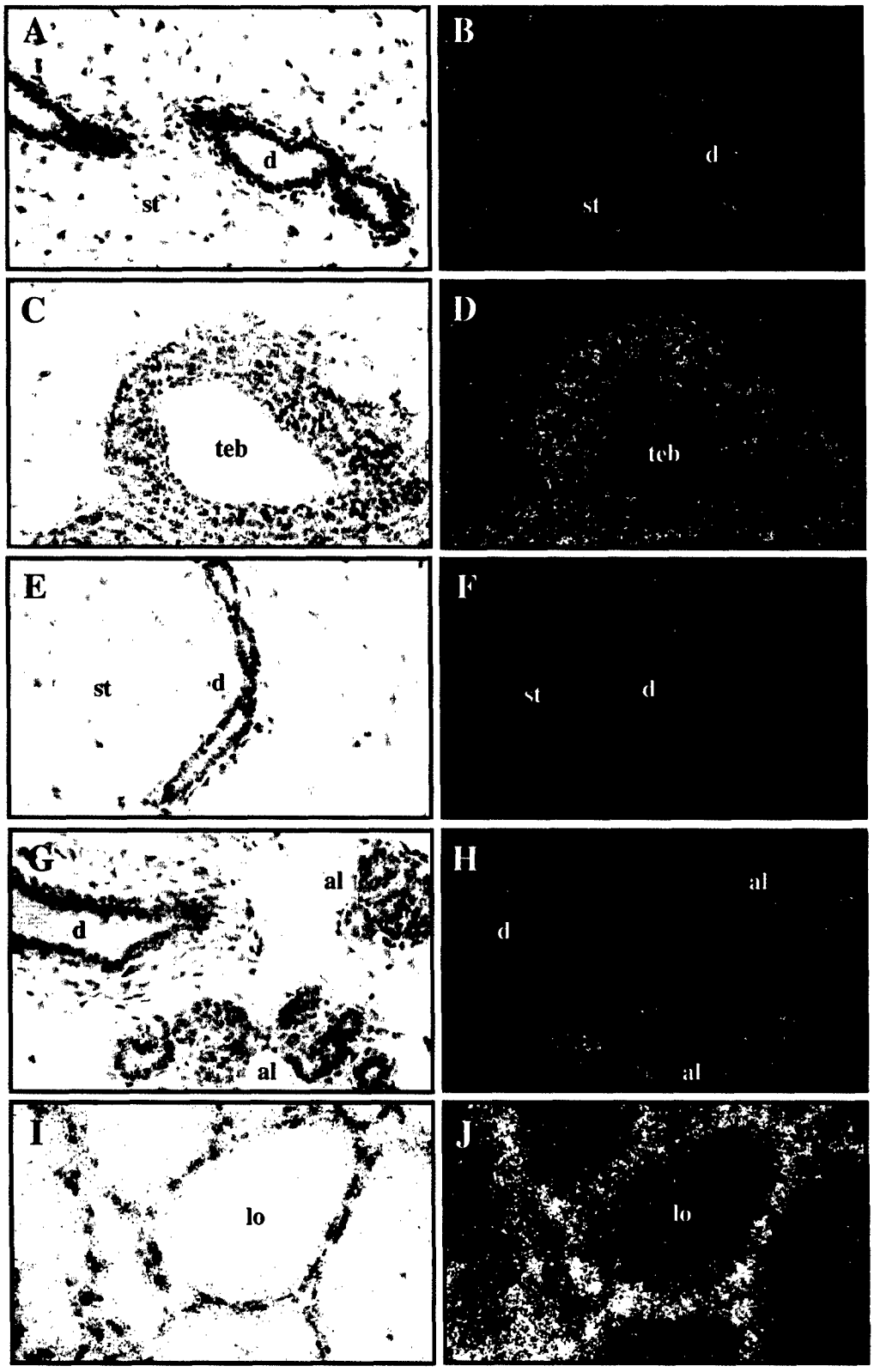

FIG. 8. Spatial expression of *Mlk1* during mammary development. *In situ* hybridization analysis of *Mlk1* expression during postnatal mammary development is shown. Bright-field (left) and dark-field (right) photomicrographs of mammary gland sections from female mice at 6 weeks of age (A–D), 16 weeks of age (E and F), day 7 of pregnancy (G and H), and day 2 of postlactational involution (I and J) hybridized with an ^{35}S-labeled *Mlk1*-specific antisense probe. No signal over background was detected in serial sections hybridized with a sense *Mlk1* probe. Exposure times were identical for all dark-field photomicrographs to illustrate changes in *Mlk1* expression during pregnancy. al, alveoli; d, duct; lo, secretory lobule; st, adipose stroma; teb, terminal end bud. Original magnification 500×.

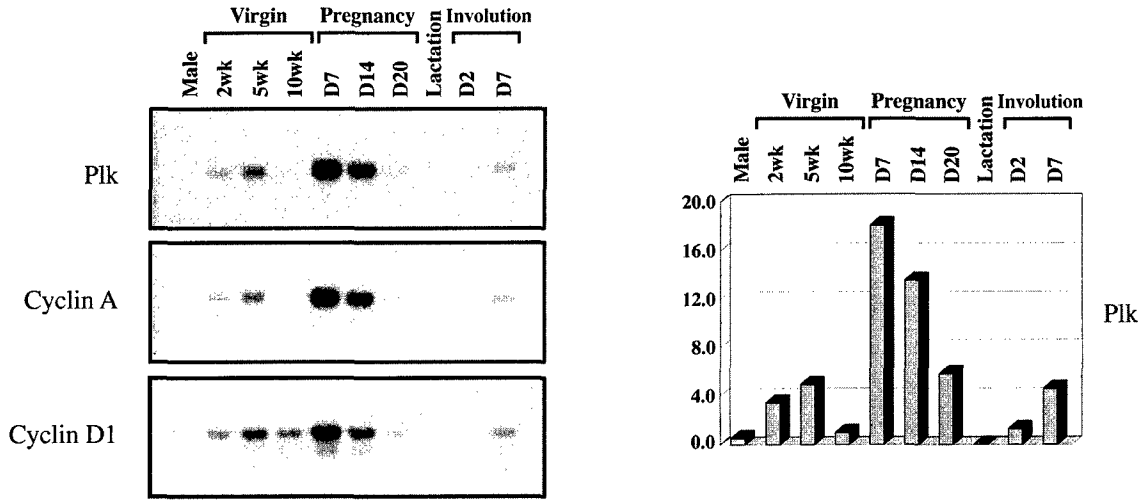

FIG. 9. Expression of protein kinases as a function of proliferation. Northern hybridization analysis of protein kinase and cyclin expression during postnatal development of the murine mammary gland is shown. 3 μg of poly(A)$^+$ RNA isolated from the mammary glands of FVB mice at the indicated time points was hybridized to ^{32}P-labeled cDNA probes for the genes indicated. Origins of the mammary developmental time points are as in Fig. 3. Phosphorimager analysis of the Northern blot for *Plk* is shown on the right. Expression levels normalized to β-actin are shown relative to adult virgin (10 wk).

pressed in epithelial cells of terminal end buds compared to ducts (Fig. 8, cf. 8B and 8D). This finding may explain the upregulation of *Mlk1* observed in the mammary glands of 5-week-old mice. *In situ* hybridization further revealed that the modest upregulation of *Mlk1* expression that occurs during pregnancy is due to preferential induction of *Mlk1* expression in developing alveoli compared to ducts, and also confirmed the dramatic upregulation of this kinase in involuting alveoli at day 2 of regression. The preferential expression of *Mlk1* in specific structures within the epithelial compartment, such as terminal end buds and developing alveoli, demonstrates that the expression of this kinase is regulated spatially as well as temporally during mammary development.

In contrast to *Tyro3* and *Mlk1*, expression of the *Csk*-related cytoplasmic tyrosine kinase *Ctk* remains low throughout virgin development and pregnancy (Fig. 7). Induction of *Ctk* expression is initially observed during lactation, with maximal upregulation occurring at day 7 of involution. *Ctk* expression has previously been described only in the brain (Brinkley *et al.*, 1995). Together, the developmental patterns of expression observed for *Tyro3*, *Mlk1*, and *Ctk* suggest that these kinases may play a role in the dramatic changes that occur in the mammary gland during involution.

Proliferation-Dependent Patterns of Kinase Expression

An intriguing pattern of developmental expression was observed for the mammalian polo-like kinase, *Plk*. *Plk* expression is maximally upregulated in the mammary gland at day 7 of pregnancy, with a progressive decline in expression observed thereafter (Fig. 9). No expression was detected during lactation. Smaller increases in *Plk* expression were noted in the mammary glands of 5-week-old nulliparous animals. The observation that the upregulation of *Plk* expression coincides with peak alveolar proliferation rates during early pregnancy, as well as previous observations that *Plk* expression is cell cycle-regulated (Lee *et al.*, 1995), suggested that the developmental pattern of expression of this kinase reflects proliferative events in the mammary gland. This hypothesis is supported by the marked similarities in the expression profiles of *Plk*, cyclin A, and cyclin D1 (Fig. 9). As such, these findings strongly suggest that the temporal profile of *Plk* expression reflects increases in mitotic activity that occur in the mammary gland during puberty and early pregnancy. Consistent with this hypothesis, the developmental expression of *Plk* in the mammary gland is spatially restricted to proliferating cellular compartments, particularly in terminal end buds during puberty and alveolar buds during pregnancy (data not shown).

Spatial Regulation of Kinase Expression

In addition to the diverse temporal patterns of expression observed for the kinases analyzed in this study, diverse spatial patterns of expression were also observed. Similar to *Mlk1* and *Plk*, the serine/threonine kinase *SLK* is upregulated in the mammary epithelium at day 7 of pregnancy (Figs. 5, 7–9, and 10A–10D, and data not shown). However, whereas *Mlk1* and *Plk* are preferentially expressed in alveoli compared to ducts at this stage of development, *SLK* upregulation occurs in both ducts and alveoli. This observation suggests that upregulation of *SLK* expression may occur in response to signals that are distributed throughout

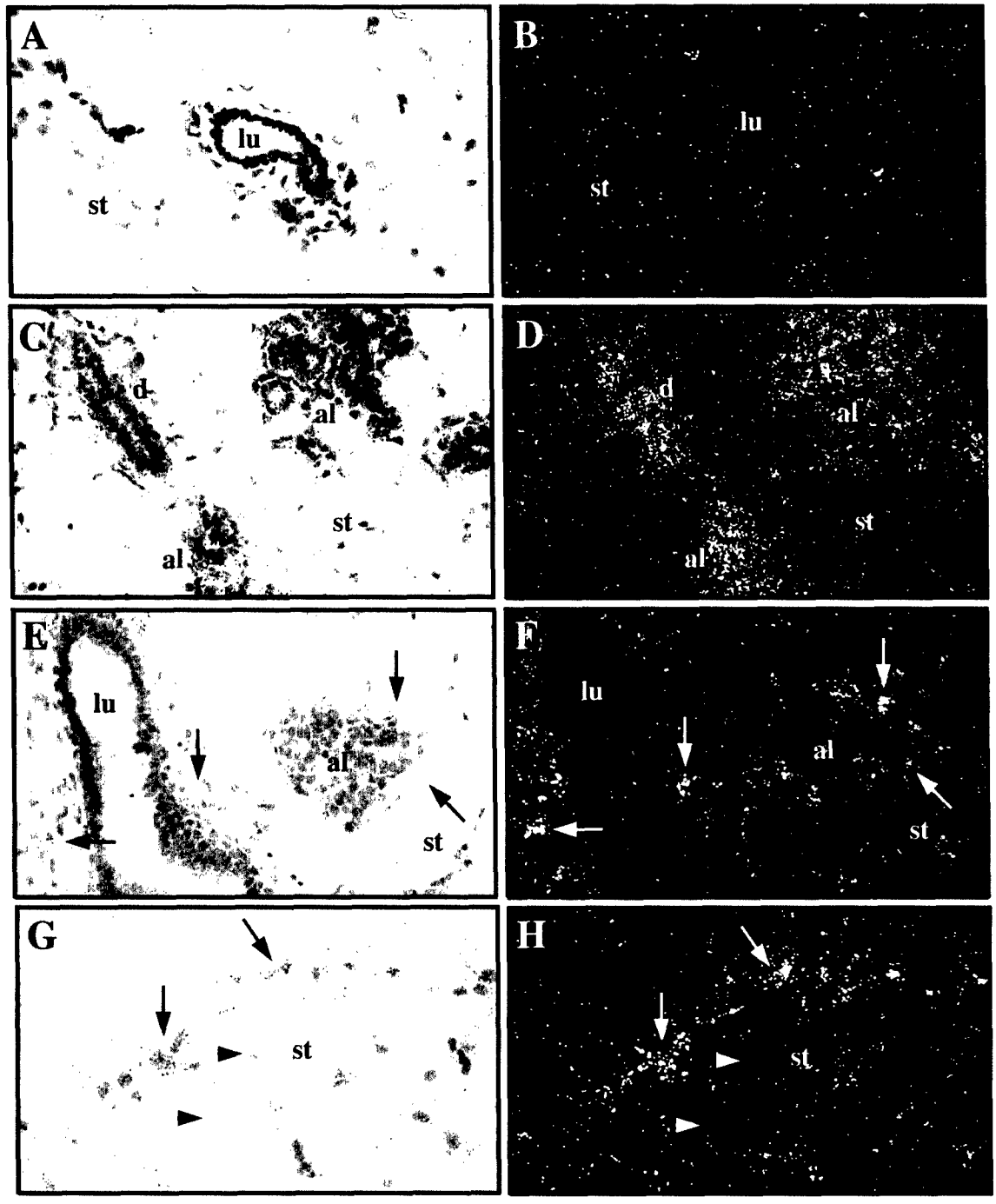

FIG. 10. Spatial expression of *SLK* and *Tyro3* during mammary development. *In situ* hybridization analysis of *SLK* (A–D) *and Tyro3* (E–H) expression during postnatal mammary development. Bright-field (left) and dark-field (right) photomicrographs of mammary gland sections from nulliparous female mice at 16 weeks of age (A and B) or day 7 of pregnancy (C–H) hybridized with ^{35}S-labeled antisense probes specific for *SLK* or *Tyro3*. Stromal cells at the edge of the mammary fat pad are shown in panels G and H. No signal over background was detected in serial sections hybridized with sense *SLK* or *Tyro3* control probes. Exposure times were identical for dark-field photomicrographs for *SLK* to illustrate changes in expression during pregnancy. al, alveoli; d, duct; lu, lumen of epithelial duct; st, adipose stroma. Arrows indicate cells expressing *Tyro3*. Cells without detectable *Tyro3* expression are indicated by arrowheads. Original magnification 500×.

the epithelium, rather than to changes specific to a subset of epithelial cells within a particular compartment.

Unlike the majority of kinases analyzed in this study, expression of the receptor tyrosine kinase *Tyro10* was restricted to the stroma of the mammary gland (Figs. 10E–10H). This cell type-specificity was predicted based on

our initial finding that *Tyro10* is preferentially expressed in cell lines that are negative for expression of the epithelial marker cytokeratin 18 (Fig. 2). Within the mammary gland, *Tyro10* is expressed in regions immediately surrounding epithelial structures (Fig. 10E and 10F) as well as in regions at the periphery of the mammary fat pad (Fig. 10G and 10H). Interestingly, *Tyro10* expression is strikingly heterogeneous in stromal cells, suggesting that the expression of this kinase may be restricted to a specific stromal cell type.

DISCUSSION

We have isolated 33 tyrosine kinases and 8 serine/threonine kinases that are expressed during the postnatal development of the murine mammary gland. Since transcription is one of the key steps at which gene expression is regulated, we chose to examine the mRNA expression of each of these kinases in a panel of transgenic mammary epithelial cell lines and in the mammary gland during multiple stages of development. Although protein kinases are typically regulated at the posttranslational level, the majority of kinases analyzed in this study were also found to be developmentally regulated at the mRNA level. Kinases were subsequently clustered into groups based on similarities in these expression patterns as a first step in drawing inferences about developmental processes in which they might be involved. In this manner, the panel of protein kinases identified in this study was used to produce a temporal map of developmental gene expression for the mammary gland.

While the temporal patterns of gene expression observed for the protein kinases surveyed in this study were diverse, application of a clustering approach revealed an ordered set of expression profiles in which successive waves of kinase expression occur during development. This finding suggests that a coordinated program of protein kinase expression exists that may play a role in regulating the cascade of events constituting mammary development.

A wide range of kinases was isolated in this study, including members of multiple receptor tyrosine kinase, cytoplasmic tyrosine kinase, and serine/threonine kinase subfamilies. A subset of the kinases that were identified in mammary gland samples was not expressed in the mammary epithelial cell lines tested, presumably as a result of their expression in a nonepithelial cell type. In contrast, virtually all of the kinases identified in mammary tumor cell lines were also found to be expressed in the mammary gland during development. This observation suggests that the expression of kinases detected in mammary epithelial cell lines is not merely a consequence of malignant transformation, nor of culture conditions, but rather suggests that these molecules may play a role in the normal physiology of the mammary gland. Of note, the kinase expression patterns observed in the panel of mammary epithelial cell lines tested were markedly heterogeneous, with dissimilar expression patterns observed even for kinases that displayed similar expression profiles during development. We conclude that kinase expression patterns in mammary gland samples and in epithelial cell lines reflect distinct aspects of mammary biology.

In recent cDNA microarray experiments in the yeast *S. cerevisiae*, more than 60% of characterized genes that were found to be regulated in a cell cycle-dependent manner were already known to have functions related to the cell cycle (Cho *et al.*, 1998). Thus, while the finding that expression of a particular kinase is developmentally regulated does not prove that the kinase plays a role in development, the spatial and temporal patterns of expression for a gene may provide important clues to its biological role. Kinases exhibiting distinctive patterns of regulation during mammary development may, in fact, be involved in controlling or mediating developmental events. Similarly, the identification of kinases that share developmental expression profiles may also identify kinases whose developmental functions are related.

The vast majority of protein kinases isolated in this screen currently have no recognized role in mammary development. Since poorly characterized genes whose expressions fluctuate in parallel may not only be regulated by parallel pathways, but also may function in parallel pathways, RNA expression patterns may provide a straightforward means of gaining insight into roles played in mammary development. As such, extrapolation may yield insights into the role in mammary development played by kinases whose functions have been elucidated in other tissues. Conversely, insight into the function of kinases lacking previously described roles in murine development may be gained by extrapolation from patterns of kinase expression during mammary development.

The expression of a number of kinases isolated in our study has previously been reported to be restricted to tissues other than the mammary gland. Such kinases include *EphB3, EphA7, Ctk, Lyn, Hck,* and *Tec.* Each of these kinases is also expressed in the mammary epithelial cell lines tested. Our observation that the majority of these kinases are developmentally regulated in the mammary gland suggests that these molecules have additional unrecognized functions. It will be of great interest to determine whether the functions of these kinases in mammary development are analogous to their functions in other tissues.

RT-PCR screens designed to identify protein kinases expressed in mammary carcinomas or in the mammary gland have previously been reported by three groups: Lehtola *et al.* isolated 10 protein kinases from the human breast cancer cell line MCF-7; Cance *et al.* isolated 25 protein kinases from a human breast carcinoma and from the human breast cancer cell line 600PEI; and Andres *et al.* isolated 24 protein kinases from murine mammary glands (Andres *et al.*, 1995; Cance *et al.*, 1993; Lehtola *et al.*, 1992). In total, these screens resulted in the identification of 43 protein kinases, 21 of which were also identified in the present study. In addition to these previously isolated kinases, our screen has resulted in the identification of an additional 20 kinases expressed in the mammary gland,

bringing the total number of kinases identified in this manner to 63.

In aggregate, beyond providing clues to the regulation of different stages of postnatal mammary development by specific protein kinases, approaches similar to those taken here should prove useful in identifying sequences of events, as well as coherent regulatory patterns, in mammary development. Moreover, by analogy with hematopoiesis, certain kinases may be expressed in the mammary gland in a lineage-restricted manner and may thereby serve as useful markers for epithelial or stromal cell subtypes. As such, it is likely that the composite spatial and temporal pattern of kinase expression in the mammary gland at any given developmental stage can provide important information regarding its physiological state and should provide insight into the molecules that regulate different stages of postnatal mammary development. Ultimately, the finding that most kinases expressed in the mammary gland are developmentally regulated suggests that the array of kinases participating in the regulation of mammary development is considerably broader than currently appreciated.

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Cloning and Characterization of *Hunk,* a Novel Mammalian SNF1-Related Protein Kinase

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We previously identified a novel protein kinase, *Hunk,* by means of a degenerate PCR screen designed to isolate kinases expressed in the murine mammary gland. We now describe the molecular cloning, chromosomal localization, and activity of this kinase and characterize its spatial and temporal pattern of expression in the mouse. We have isolated a 5.0-kb full-length cDNA clone that contains the 714-amino-acid open reading frame encoding Hunk. Analysis of this cDNA reveals that Hunk is most closely related to the SNF1 family of serine/threonine kinases and contains a newly described SNF1 homology domain. Accordingly, antisera specific for Hunk detect an 80-kDa polypeptide with associated phosphotransferase activity. *Hunk* is located on distal mouse chromosome 16 in a region of conserved synteny with human chromosome 21q22. During fetal development and in the adult mouse, *Hunk* mRNA expression is developmentally regulated and tissue-specific. Moreover, *in situ* hybridization analysis reveals that *Hunk* expression is restricted to subsets of cells within a variety of organs in the adult mouse. These findings suggest a role for Hunk in murine development. © 2000 Academic Press

INTRODUCTION

Major insights into the molecular mechanisms of differentiation, development, and carcinogenesis have been obtained through studies of protein kinases in a wide range of biological systems. The finding that aberrantly regulated or aberrantly functioning protein kinases can disrupt normal developmental processes or promote carcinogenesis illustrates the fact that phosphorylation events play critical roles in the regulation of cell growth and differentiation. In addition, some protein kinases are expressed in a lineage-specific manner and are thereby useful markers for defining cellular subtypes (Dymecki *et al.,* 1990; Mischak *et al.,* 1991; Rawlings and Witte, 1994; Schnurch and Risau, 1993; Siliciano *et al.,* 1992; Valenzuela *et al.,* 1995).

The key role played by serine/threonine kinases in regulating diverse cellular processes is exemplified by studies of SNF1-related kinases. Several members of the SNF1 family of kinases function in signal transduction pathways involved in the cellular response to nutritional or environmental stresses (Hardie *et al.,* 1994). The *Saccharomyces cerevisiae* protein kinase, SNF1, and its mammalian counterpart, AMP-activated protein kinase (AMPK), function in highly conserved signal transduction pathways that promote energy conservation. SNF1 family members have also been implicated in a variety of developmental processes including the regulation of cellular proliferation and differentiation. These include *Msk* in murine cardiac development, SNRK in adipocyte differentiation, C-TAK1 in cell cycle control, and the *Caenorhabditis elegans* SNF-1 related kinase, PAR-1, in the establishment of embryonic polarity (Becker *et al.,* 1996; Guo and Kemphues, 1995; Peng *et al.,* 1998; Ruiz *et al.,* 1994). In fact, SNF-1 itself has been found to mediate cell cycle arrest in response to starvation (Thompson-Jaeger *et al.,* 1991). Thus, members of the SNF1 kinase family have been demonstrated to regulate a variety of important cellular processes.

In light of the importance of protein kinases in development and carcinogenesis, we previously performed a degenerate PCR-based screen aimed at identifying protein kinases expressed in the murine mammary gland during development and in mammary epithelial cell lines derived from different transgenic mouse models of breast cancer (Chodosh *et al.,* 1999, submitted for publication; Gardner *et al.,* in press; Stairs *et al.,* 1998). In the course of these studies, we identified a cDNA encoding a catalytic domain fragment from a novel protein kinase, *Hunk.* In this report, we show that *Hunk* encodes an 80-kDa polypeptide

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associated with phosphotransferase activity. *Hunk* is evolutionarily conserved, being most closely related to the SNF1 family of serine/threonine kinases, and is located on mouse chromosome 16 in a region of conserved synteny with human chromosome 21q22. *Hunk* expression in the mouse is developmentally regulated and tissue-specific. Interestingly, within several tissues in the adult mouse, *Hunk* is expressed in a heterogeneous pattern, suggesting that the expression of this kinase is restricted to particular subtypes of cells within a variety of tissues.

MATERIALS AND METHODS

Cloning of a full-length Hunk cDNA. Poly(A)$^+$ RNA isolated from the MMTV-*Ha-ras* transgenic mammary epithelial tumor cell line, AC816 (Morrison and Leder, 1994), or from FVB mouse embryos harvested at day 14 of gestation was used to generate independent cDNA libraries using either the Uni-ZAP (AC816) or the Zap Express (day 14 embryo) lambda phage vector (Stratagene) according to the manufacturer's instructions. A total of 5×10^5 plaques from each library were screened by standard methods using a [α-^{32}P]dCTP-labeled random-primed cDNA probe (BMB Random Prime). The catalytic domain fragment corresponding to nucleotides 618 to 824 of *Hunk* was used to screen two independently generated AC816 cDNA libraries. The day 14 mouse embryo cDNA library was subsequently screened using cDNA fragments corresponding to nucleotides 132 to 500 and 276 to 793 of *Hunk*. Hybridization was performed at a concentration of 10^6 cpm/ml in 48% formamide, 10% dextran sulfate, 4.8× SSC, 20 mM Tris (pH 7.5), 1× Denhardt's solution, 20 μg/ml salmon sperm DNA, and 0.1% SDS at 42°C overnight. Following hybridization, blots were washed in 2× SSC/0.1% SDS at room temperature (RT) for 30 min (×2), followed by 0.2× SSC/0.1% SDS at 50°C for 20 min (×2), and subjected to autoradiography (Kodak XAR-5). Positive phage clones were plaque purified, and plasmids were liberated by *in vivo* excision according to the manufacturer's instructions (Stratagene). From each library, the independent clone with the largest insert size was completely sequenced by automated sequencing using an ABI Prism 377 DNA sequencer. The full-length *Hunk* cDNA sequence has been deposited with the GenBank database (Accession No. AF167987).

Sequence analysis. Sequence analysis including predicted open reading frames and calculation of predicted molecular weights was performed using MacVector (Oxford Molecular Group). Pairwise and multiple sequence alignments of kinase catalytic domains were performed using the ClustalW alignment program BLOSUM series with an open gap penalty of 10, an extend gap penalty of 0.05, and a delay divergent of 40%. Multiple sequence alignment and phylogenetic calculations were performed using the ClustalX multisequence alignment program with the same parameters as above. DendroMaker 4.0 was used to draw an unrooted phylogenetic tree.

Tissue preparation. FVB mouse embryos were harvested at specified time points following timed matings. Day 0.5 postcoitus was defined as noon of the day on which a vaginal plug was observed. Tissues used for RNA preparation and protein extracts were harvested from 15- to 16-week-old virgin mice and snap frozen on dry ice. Tissues used for *in situ* hybridization analysis were embedded in OCT compound.

Northern analysis. RNA was prepared by homogenization of snap-frozen tissue samples or tissue culture cells in guanidinium isothiocyanate supplemented with 7 μl/ml of 2-mercaptoethanol followed by ultracentrifugation through cesium chloride as previously described (Marquis *et al.*, 1995; Rajan *et al.*, 1997). Poly(A)$^+$ RNA was selected using oligo(dT) cellulose (Pharmacia), separated on a 0.7% LE agarose gel, and passively transferred to a GeneScreen membrane (NEN). Northern hybridization was performed as described using a ^{32}P-labeled cDNA probe encompassing nucleotides 1149 to 3849 of *Hunk* generated by random-primed labeling (BMB) (Marquis *et al.*, 1995). Hybridization was carried out as detailed above for cDNA library screening.

In vitro transcription/translation. *In vitro* transcription and translation were performed on 1 μg of plasmid DNA using rabbit reticulocyte lysates in the presence of either [^{35}S]Met or unlabeled methionine according to the manufacturer's instructions (TNT kit, Promega). Completed reactions were electrophoresed on a 10% SDS–PAGE gel and were subjected either to autoradiography or to immunoblotting as described below.

Generation of anti-Hunk antisera. GST–Hunk recombinant fusion proteins containing amino-teminal (amino acids 32–213) or carboxyl-terminal (amino acids 556–714) regions of Hunk were expressed in BL21 bacterial cells and purified using glutathione–Sepharose beads according to the manufacterer's instructions (Pharmacia). Following removal of the GST portion by cleavage with Prescission Protease (Pharmacia), the liberated carboxyl-terminal Hunk polypeptide was further purified by isolation on a 15% SDS–PAGE gel. Purified Hunk polypeptides were injected into rabbits (Cocalico Biologicals) in cleavage buffer (amino-terminal) or embedded in acrylamide gel slices (carboxyl-terminal). Antisera were affinity-purified on cyanogen bromide-coupled Sepharose columns crosslinked with their respective antigens according to the manufacturer's instructions (Pharmacia). Bound antibodies were then eluted sequentially with 100 mM glycine, pH 2.5, and 100 mM triethylamine, pH 11.5, and neutralized with 1/10 vol of 1.0 M Tris (pH 7.5) (Harlow and Lane, 1999).

Immunoblotting analysis. Protein extracts were generated by lysing tissue culture cells or homogenizing murine mammary glands in EBC buffer composed of 50 mM Tris (pH 7.9), 120 mM NaCl, and 0.5% NP-40 supplemented with 1 mM β-glycerol phosphate, 50 mM NaF, 20 μg/ml aprotinin, 100 μg/ml Pefabloc (BMB), and 10 μg/ml leupeptin. Equivalent amounts of each extract were electrophoresed on 10% SDS–PAGE gels and transferred overnight onto nitrocellulose membranes. Following visualization by Ponceau staining to verify equal protein loading and even transfer, membranes were incubated with blocking solution consisting of 4% dry milk, 0.05% Tween 20, and 1× phosphate-buffered saline (PBS) at RT. Primary antibody incubation with affinity-purified antisera was performed at RT for 1 h at a final concentration of approximately 2 μg/ml in blocking solution. Following three RT washes in blocking solution, blots were incubated with a 1:10,000 dilution of a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch) for 30 min at RT. Following three washes in blocking solution and two washes in 1× PBS, blots were developed using the ECL Plus system according to the manufacturer's instructions (Amersham Pharmacia) followed by exposure to film (Kodak XAR-5).

Immunoprecipitation of Hunk. Protein was extracted from tissue culture cells by lysis in EBC buffer for 15 min at 4°C. From each extract, 500 μg of protein in 250 μl of EBC was precleared with 40 μl of 1:1 Protein A-Sepharose:PBS for 3 h at 4°C. Precleared lysates were incubated overnight at 4°C with affinity-purified antisera raised against the amino-terminus of Hunk (3 μg), the carboxyl-terminus of Hunk (0.1 μg), or polypeptides unrelated to Hunk (0.1 or 3 μg). Immune complexes were precipitated by incubating with 40 μl of 1:1 Protein A-Sepharose:PBS for 3 h at 4°C. Complexes were washed twice with PBS, washed once with EBC, and electrophoresed on a 10% SDS–PAGE gel. Following transfer onto nitrocellulose membranes immunoblotting was performed as described above.

Kinase assay. Protein was extracted from snap-frozen lactating murine mammary glands and from 8Ma1a cells (Morrison and Leder, 1994) by dounce homogenization in EBC buffer containing protease inhibitors. Extracts containing 820 μg protein in 1 ml EBC were precleared with 40 μl 1:1 Protein A-Sepharose:PBS (Pharmacia) for 1 h at 4°C. One-quarter of the precleared lysate was incubated at 4°C overnight with 1.2 μg/ml of affinity-purified antisera raised against the amino-terminus of Hunk. Immune complexes were precipitated with 40 μl of 1:1 Protein A-Sepharose:PBS. *In vitro*

kinase activity of the resulting immunoprecipitates was assayed under final reaction conditions consisting of 20 mM Tris (pH 7.5), 5 mM $MgCl_2$, 100 μM dATP, 0.5 μCi/μl [γ-^{32}P]ATP, and 0.15 μg/μl histone H1 for 45 min at 37°C. Reactions were electrophoresed on a 15% SDS–PAGE gel and were subjected to autoradiography.

RNase protection analysis. Ribonuclease protection analysis was performed as described (Marquis *et al.,* 1995). Body-labeled antisense riboprobes were generated using linearized plasmids containing nucleotides 276 to 500 of the *Hunk* cDNA and 1142 to 1241 of *β-actin* (GenBank Accession No. X03672) using [α-^{32}P]UTP and the Promega *in vitro* transcription system with T7 polymerase. The β-actin antisense riboprobe was added to each reaction as an internal control. Probes were hybridized with RNA samples at 58°C overnight in 50% formamide/100 mM Pipes (pH 6.7). Hybridized samples were digested with RNase A and T1, purified, electrophoresed on a 6% denaturing polyacrylamide gel, and subjected to autoradiography.

In situ hybridization. In situ hybridization was performed as described (Marquis *et al.,* 1995). Antisense and sense probes were synthesized with the Promega *in vitro* transcription system using ^{35}S-UTP and ^{35}S-CTP from the T7 and SP6 RNA polymerase promoters of a PCR template containing sequences corresponding to nucleotides 276 to 793 of *Hunk,* a region demonstrated to recognize both mRNA transcripts. Exposure times were 6 weeks in all cases.

Interspecific mouse backcross mapping. Interspecific backcross progeny were generated by mating (C57BL/6J × *Mus spretus*)F_1 females and C57BL/6J males as described (Copeland and Jenkins, 1991). A total of 205 N_2 mice were used to map the *Hunk* locus (see below for details). DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern transfer, and hybridization were performed essentially as described (Jenkins *et al.,* 1982). All blots were prepared with Hybond-N$^+$ nylon membrane (Amersham). A 520-bp *Eco*RI fragment corresponding to nucleotides 276 to 793 of the *Hunk* cDNA was labeled with [α-^{32}P]dCTP using a nick-translation labeling kit (Boehringer Mannheim). Washing was performed at a final stringency of 1.0× SSCP/0.1% SDS at 65°C. A major fragment of 6.9 kb was detected in *Sac*I-digested C57BL/6J DNA, and a major fragment of 5.8 kb was detected in *Sac*I-digested *M. spretus* DNA. The presence or absence of the 5.8-kb *Sac*I *M. spretus*-specific fragment was followed in backcross mice.

A description of the probes and RFLPs for the loci linked to *Hunk* including *App, Tiam1,* and *Erg* has been reported previously (Fan *et al.,* 1996). Recombination distances were calculated using Map Manager, version 2.6.5. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

RESULTS

We previously performed a screen designed to detect protein kinases expressed in the murine mammary gland and in breast cancer cell lines to identify regulatory molecules potentially involved in mammary development and carcinogenesis. RT-PCR with degenerate oligonucleotide primers was employed to amplify catalytic subdomains of protein kinases expressed in six murine breast cancer cell lines or in the murine mammary gland during various stages of development (Chodosh *et al.,* 1999, submitted for publication; Gardner *et al.,* in press; Stairs *et al.,* 1998). Individual PCR products were subcloned and screened by a combination of DNA sequencing and colony-lift hybridization. Examination of approximately 1500 cDNA clones from this screen resulted in the identification of 41 protein kinases including 33 tyrosine kinases and 8 serine/threonine kinases, 3 of which were novel.

One of these novel putative serine/threonine kinases, originally referred to as *Bstk1,* was first identified as a 207-bp RT-PCR product isolated from a mammary epithelial cell line derived from an adenocarcinoma arising in an MMTV-*neu* transgenic mouse (Chodosh *et al.,* 1999, submitted for publication). The cDNA encoding *Bstk1* was subsequently found to be upregulated in the mammary gland during early pregnancy and following treatment with 17β-estradiol and progesterone. In addition, *Bstk1* was found to be preferentially expressed in mammary tumor cell lines derived from MMTV-*neu* as compared with MMTV-*c-myc* transgenic mice (Gardner *et al.,* submitted for publication). Based upon this expression pattern, *Bstk1* was renamed *Hunk,* for hormonally upregulated, *neu*-tumor-associated kinase.

Isolation of cDNA Clones Encoding Hunk

To isolate the full-length mRNA transcript from which *Bstk1* was derived, the initial 207-bp RT-PCR product was used to screen a cDNA library prepared from the *H-ras* transformed mammary epithelial cell line, AC816 (Morrison and Leder, 1994). A cDNA probe derived from the 5′ end of the longest clone isolated, G3, was subsequently used to screen a day 14 murine embryonic cDNA library. Six additional nonchimeric cDNA clones ranging in length from 4.4 to 5.0 kb were isolated from this library; each of the clones possessed a poly(A) tail and a restriction pattern similar to that of G3 (data not shown). Dideoxy sequencing of the 5′ and 3′ termini of these clones in addition to restriction mapping revealed that all seven cDNA clones were contiguous. The longest cDNA clones isolated from each library, G3 and E8, were completely sequenced on both strands (Fig. 1). Comparison of the 5024-nt sequence of clone E8 with that of clone G3 revealed that clone E8 contains an additional 40 nucleotides at its 5′ end and that the length of a poly(T) tract in the 3′-untranslated region (UTR) of the two clones differs by a single nucleotide. There were no additional differences between these two clones.

The 5024-nucleotide sequence of clone E8, hereafter referred to as *Hunk,* contains the entire 207-bp RT-PCR fragment, *Bstk1,* from positions 618 to 824 (Fig. 1). *Hunk* possesses an open reading frame (ORF) 2142 nucleotides in length beginning with a putative initiation codon at nucleotide 72. Comparison of the nucleotide sequence surrounding this site with the Kozak (1987, 1991) consensus sequence, $GCC^A/_GCCAUGG$, reveals matches at positions −4, −3, and −2. The nucleotide sequence of the 5′-UTR and the first 100 nt of the *Hunk* ORF are extremely GC-rich (>80%). Other genes bearing such GC-rich sequences have been found to be subject to translational control (Kozak, 1991). The 3′-UTR of *Hunk* is 2.8 kb in length and lacks a canonical AATAAA polyadenylation signal, containing instead the relatively uncommon signal, AATACA, 18 nucleotides upstream of the poly(A) tract (Bishop *et al.,*

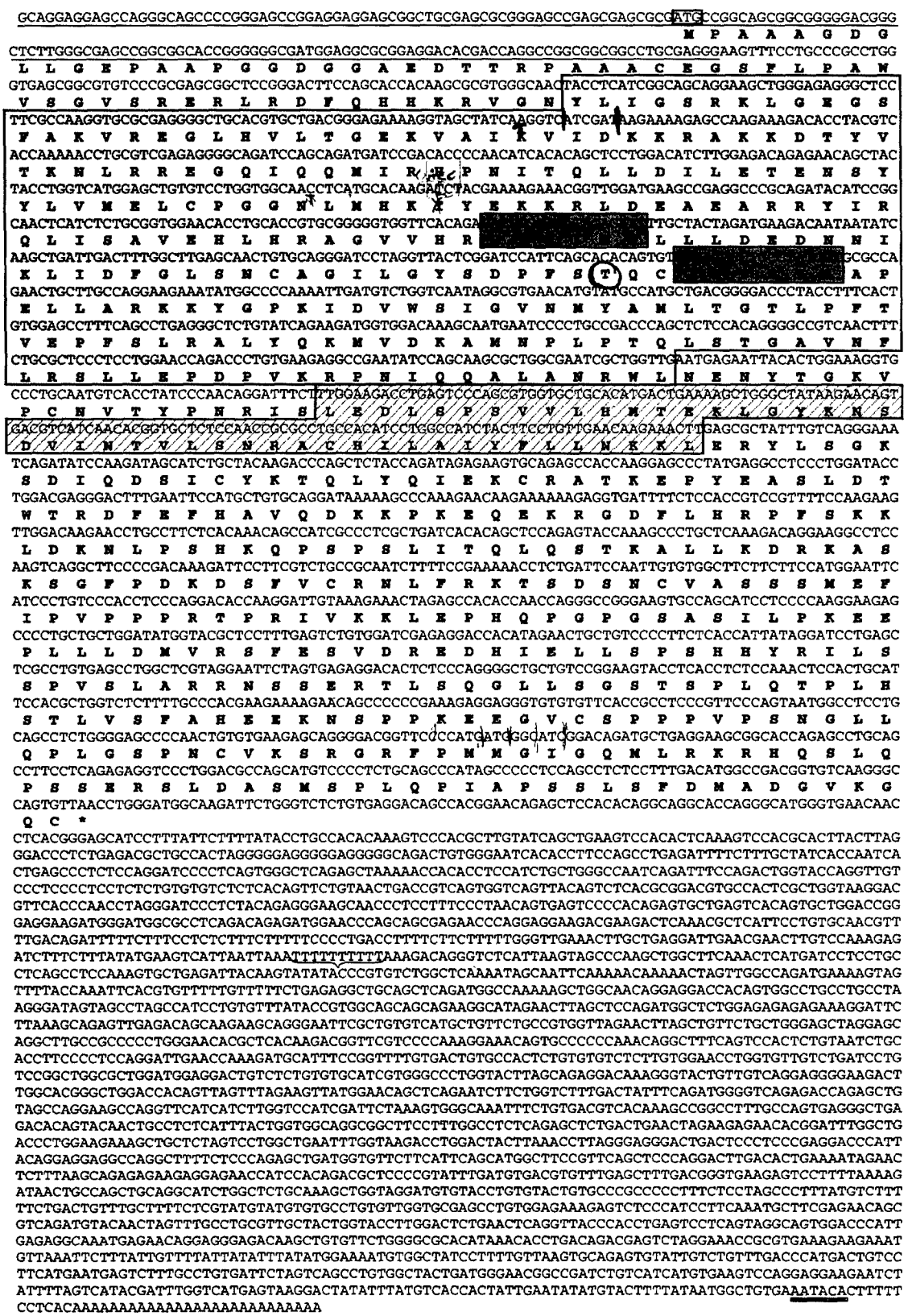

FIG. 1. Nucleotide and deduced amino acid sequence of *Hunk*. The composite nucleic acid sequence and conceptual translation of full-length *Hunk* cDNA are shown. Nucleotide coordinates are shown on the left. Amino acid coordinates are shown in boldface type on the right. A light shaded box indicates the kinase catalytic domain. Dark shaded boxes denote amino acid motifs characteristic of serine/threonine kinases. The SNF1 homology region, SNH, is denoted by a hatched box. The GC-rich region in the 5′-UTR and the putative polyadenylation sequence in the 3′-UTR are underlined by thin and thick lines, respectively. An asterisk denotes the stop codon. A bracket in the 3′ UTR denotes the poly(T) tract, which differs in length between the two independent cDNA clones (clone E8 is shown here).

1986; Herve *et al.,* 1995; Myohanen *et al.,* 1991, 1994; Parthasarathy *et al.,* 1997; Tokishita *et al.,* 1997).

While this work was in progress, a 588-nucleotide portion of the catalytic domain of *Hunk* was independently isolated by another group and shown to recognize an mRNA approximately 4 kb in length (Korobko *et al.,* 1997). Subsequently, this same group deposited a 5026-nt full-length sequence in GenBank (Accession No. AF055919) that is 10 nucleotides shorter at the 5′ end and 98% identical to *Hunk.* No additional information is available regarding the cloning, localization, function, or *in vivo* expression of this molecule.

The conceptual ORF of *Hunk* comprises 714 amino acids and encodes a polypeptide of predicted molecular mass 79.6 kDa. This polypeptide can be divided into an amino-terminal domain of 60 amino acids, a 260-amino-acid kinase catalytic domain, and a 394-amino-acid carboxyl-terminal domain. The carboxyl-terminal domain of Hunk contains a 46-amino-acid conserved motif located 18 amino acids C-terminal to the catalytic domain that is homologous to the previously described SNF1 homology region or SNH (Becker *et al.,* 1996). The 330 amino acids carboxyl-terminal to the SNH lack homology to other known proteins.

The putative catalytic domain of Hunk contains each of the invariant amino acid motifs characteristic of all protein kinases as well as sequences specific to serine/threonine kinases (Hanks and Quinn, 1991; Hanks *et al.,* 1988). In particular, the DLKPEN motif in subdomain VIB of the *Hunk* cDNA predicts serine/threonine kinase specificity (ten Dijke *et al.,* 1994). *Hunk* also contains the serine/threonine consensus sequence, G(T/S)XX(Y/F)X, in subdomain VIII N-terminal to the APE motif conserved among all protein kinases. In addition, several amino acids in subdomains I, VII, VIII, IX, X, and XI that are conserved among tyrosine kinases are absent from the *Hunk* ORF. Thus, primary sequence analysis strongly suggests that *Hunk* encodes a functional serine/threonine kinase.

To determine whether the length of the cDNA clone encoding Hunk is consistent with the size of the *Hunk* mRNA message, Northern hybridization was performed on poly(A)$^+$ RNA isolated from a *Hunk*-expressing mammary epithelial cell line (Fig. 2A). This analysis revealed a predominant mRNA transcript 5.1 kb in length, as well as a less abundant transcript approximately 5.6 kb in length, suggesting that clone E8 may correspond to the shorter *Hunk* mRNA transcript.

The finding that all six cDNA clones isolated from a cDNA library generated from mRNA containing both 5.1- and 5.6-kb *Hunk* mRNA species contain poly(A) tails and are colinear suggests that the 5.6-kb transcript may contain additional 5′ or 3′ sequence relative to our longest cDNA clone. Consistent with this supposition is the observation that insertions or deletions relative to our *Hunk* cDNA sequence were not detected using using multiple PCR primer pairs to perform RT-PCR on first-strand cDNA prepared from RNA containing both transcripts (data not shown). The failure to identify cDNA clones containing additional 5′ sequence may be related to the GC-rich nature of the 5′ UTR of *Hunk* and the tendency of reverse transcriptase to terminate prematurely in such regions. Alternately, the difference in size between the 5.1- and the 5.6-kb transcripts may be due to utilization of an alternate downstream polyadenylation site during mRNA processing.

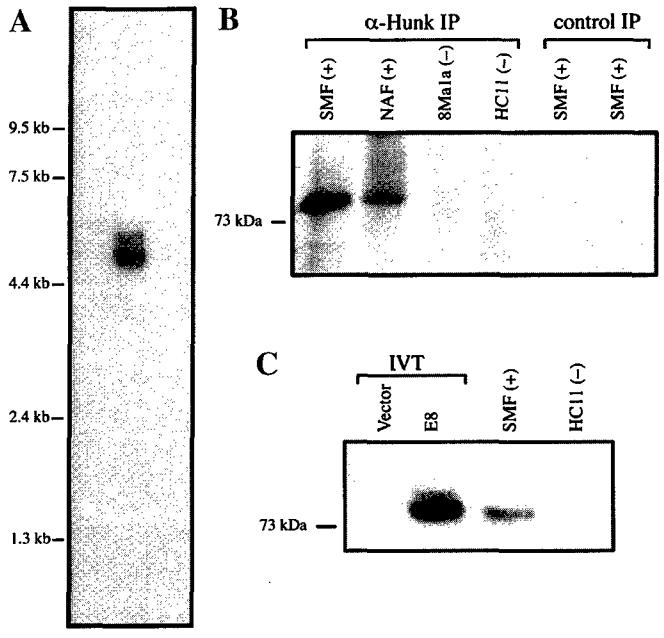

FIG. 2. Expression, identification, and coding potential of *Hunk.* (**A**) Northern hybridization analysis of 1 μg poly(A)$^+$ RNA from NAF mammary epithelial cells hybridized with a cDNA probe specific for Hunk. The relative migration of RNA size markers is indicated. (**B**) Immunoprecipitation of Hunk. Antisera raised against the amino-terminus of Hunk (α-Hunk IP) or against polypeptides unrelated to Hunk (control IP) were used to immunoprecipitate protein from lysates prepared from cells that either express (+) or do not express (−) *Hunk* mRNA. Immunoprecipitated protein was immunoblotted with antisera raised against the carboxyl-terminus of Hunk. (**C**) Immunoblotting analysis of Hunk protein using antisera raised against the carboxyl-terminus of Hunk. IVT reactions were performed in rabbit reticulocyte lysates in the presence of unlabeled methionine using either plasmid control (vector) or full-length Hunk cDNA (E8) as a template. IVT reaction products were resolved by SDS–PAGE along with lysates from *Hunk*-expressing (+) and non-expressing (−) cell lines. Note that the *in vitro* translated product detected with anti-Hunk antisera comigrates with the endogenous form of Hunk protein. The relative migration of the closest molecular weight marker is indicated.

To confirm the coding potential of the *Hunk* cDNA, *in vitro* transcription and translation of clone E8 were performed in the presence of [^{35}S]Met. This yielded an 80-kDa labeled polypeptide species, consistent with the 79.6-kDa predicted size of Hunk (data not shown), suggesting that the predicted initiation codon at nucleotide 72 is capable of functioning as a translation initiation site.

Detection of Hunk in Mammalian Cells

To detect the polypeptide encoded by the *Hunk* locus, anti-Hunk antisera were raised against recom-

binant proteins encoding amino-teminal (amino acids 32–213) and carboxyl-terminal (amino acids 556–714) regions of Hunk. Each of the antisera raised against the amino- and carboxyl-termini of Hunk identifies a polypeptide of approximately 80 kDa present in extracts from mammary epithelial cells that express *Hunk* mRNA, but not in extracts from mammary epithelial cells that do not (Fig. 2C; and data not shown). To demonstrate that this 80-kDa polypeptide corresponds to Hunk, protein extracts prepared from two mammary epithelial cell lines that express *Hunk* mRNA and from two mammary epithelial cell lines that do not express *Hunk* mRNA were subjected to immunoprecipitation/immunoblotting protocols (Fig. 2B). Immunoprecipitation of Hunk using antisera raised against the amino-terminus of Hunk, followed by immunoblotting with antisera raised against the carboxyl-terminus of Hunk, identified an 80-kDa polypeptide only in extracts prepared from cells that express *Hunk* mRNA (Fig. 2B). Similarly, immunoprecipitation of Hunk using antisera raised against the carboxyl-terminus of Hunk, followed by immunoblotting with antisera raised against the amino-terminus of Hunk, also identified an 80-kDa polypeptide only in extracts prepared from cells that express *Hunk* mRNA (data not shown). The 80-kDa polypeptide was not detected when immunoblotting was performed on immunoprecipitates prepared from *Hunk*-expressing cells when immunoprecipitation was carried out using either of two control affinity-purified antisera (Fig. 2B; and data not shown). We conclude that this 80-kDa polypeptide represents the endogenous Hunk gene product in these mammary epithelial cell lines.

To prove that clone E8 encodes the predominant form of Hunk found in mammary epithelial cells, we determined whether the *in vitro* translated product of clone E8 comigrates with endogenous Hunk. Immunoblotting of protein extracts prepared from the *Hunk* mRNA-expressing mammary epithelial cell line SMF and from rabbit reticulocyte lysates programmed with sense RNA prepared by *in vitro* transcription of clone E8 identified comigrating 80-kDa polypeptides (Fig. 2C). No band was detected in reticulocyte lysates programmed with an empty vector or in whole-cell lysates from a cell line that does not express *Hunk* mRNA. The observation that the 80-kDa polypeptide identified by anti-Hunk antisera comigrates with the polypeptide obtained following *in vitro* transcription and translation of clone E8 strongly suggests that clone E8 contains the entire ORF encoding the predominant form of *Hunk* found in mammary epithelial cells. Nevertheless, due to the absence of in-frame stop codons upstream of the putative translation initiation codon, the possibility that additional 5′ coding sequence exists cannot be excluded.

Predicted Structure and Homology to Previously Isolated Protein Kinases

Multiple sequence alignment was used to determine the homology between the kinase catalytic domain of Hunk and other previously isolated protein kinases (Fig. 3A). This analysis revealed that Hunk displays highest homology to the *S. cerevisiae* SNF1 family of serine/threonine kinases. The SNF1 family of protein kinases is composed of at least two subfamilies. The first subfamily includes SNF1 and its plant homologues including NPK5, AKin10, BKIN12, and Rkin1 as well as the mammalian SNF1 functional homologue, AMPK (Alderson *et al.,* 1991; Carling *et al.,* 1994; Le Guen *et al.,* 1992; Muranaka *et al.,* 1994). More recently, additional mammalian SNF1-related kinases have been identified that define a second subfamily. These include C-TAK1/p78, MARK1, MARK2/Emk, SNRK, and Msk, as well as the *C. elegans* kinase, PAR-1 (Becker *et al.,* 1996; Drewes *et al.,* 1997; Peng *et al.,* 1997, 1998; Ruiz *et al.,* 1994). Less closely related to either subfamily are Wpk4, Melk, and KIN1, SNF1-related kinases found in wheat, mice, and *Schizosaccharomyces pombe,* respectively (Heyer *et al.,* 1997; Levin and Bishop, 1990; Sano and Youssefian, 1994). Similar to these more distantly related SNF1 kinases, Hunk does not appear to belong to a previously defined SNF1 subfamily. Thus, based upon homology within the kinase domain, *Hunk* appears to represent a new branch of the SNF1 family tree.

Outside of a conserved kinase catalytic domain, SNF1-related protein kinases contain a region of homology referred to as the SNH or SNF1 homology domain (Becker *et al.,* 1996). Although amino acids in this motif are conserved, the functional significance of the SNH domain is unknown. Multiple sequence alignment confirms the presence of the SNH in all SNF1 family members shown in Fig. 3A and permits refinement of the conserved features of this domain (Fig. 3B). This analysis reveals that the SNH is anchored approximately 20 amino acids carboxyl-terminal to the kinase domain, spans approximately 45 amino acids, and extends further toward the amino terminus than previously reported. Our consensus identifies amino acids exhibiting greater than 70% conservation among the SNF1 family members shown as well as residues that are specific for particular SNF1 kinase subfamilies.

Although most conserved residues are shared among all family members, some residues are relatively specific for a particular subfamily. For example, the consensus amino acid at position 32 of the SNH is glutamine in subfamily I SNF1 kinases and tyrosine in subfamily II kinases. Subclass-specific residues are also found at positions 37 (A versus V) and 45 (K/R versus N). More distantly related SNF1 family members such as Wpk4 and KIN1 also have SNH domains, though the degree of homology is lower and in some cases the spacing is not conserved. Outside of its kinase and SNH domains, Hunk displays no detectable homol-

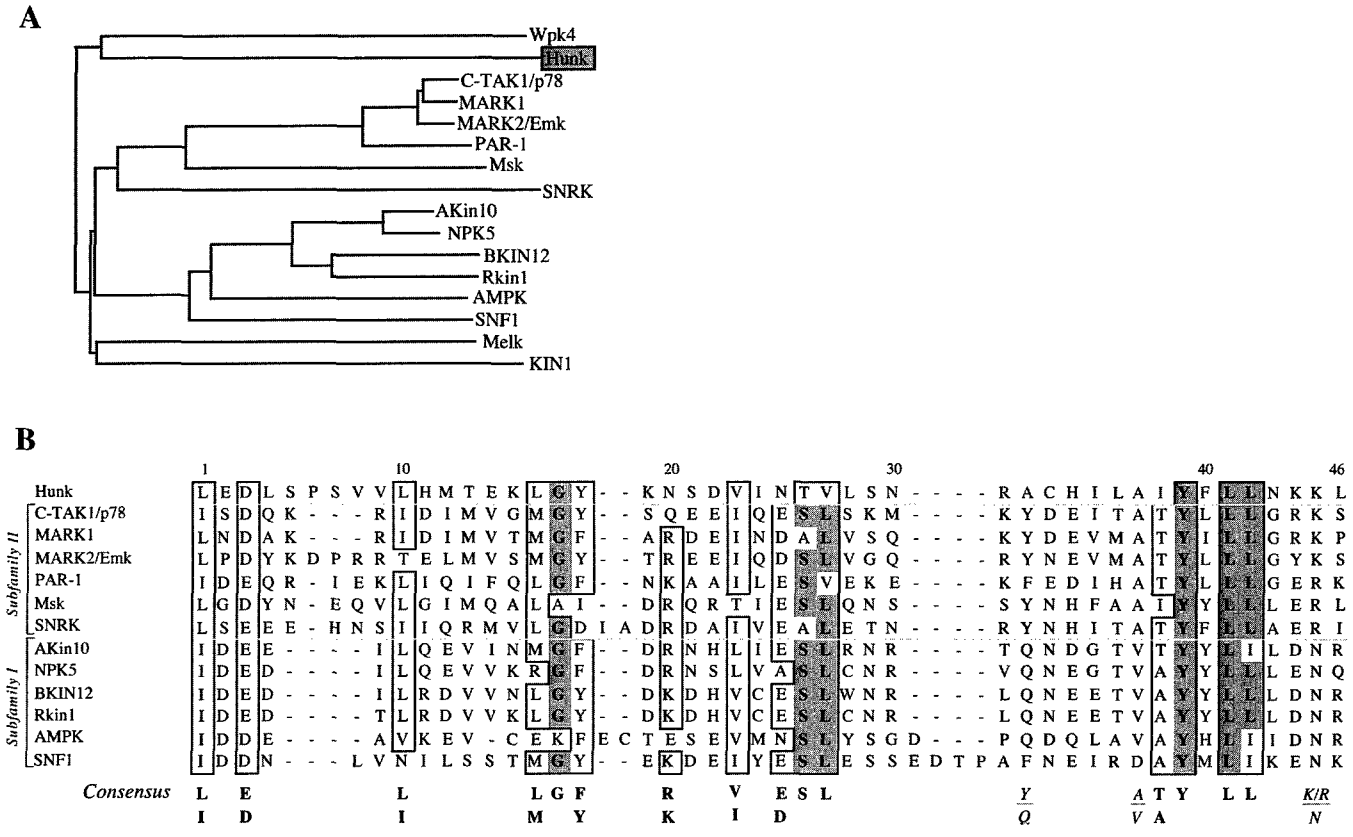

FIG. 3. Hunk represents a member of a novel subfamily of SNF1-related serine/threonine kinases. (**A**) Phylogenetic tree illustrating the relationship of Hunk kinase catalytic subdomains I–XI to other SNF1 family members. Analysis of results was performed using the ClustalX multisequence alignment program, and results were depicted using DendroMaker 4.0. (**B**) Amino acid alignment of SNF1 family members demonstrating conserved residues in the SNF homology domain. Positions at which an amino acid occurs with greater than 70% frequency are indicated in boldface type with dark shading. Positions at which similar amino acids occur with greater than 70% frequency are shown with light shading. A consensus sequence for all conserved residues is shown in boldface type at the bottom. Residues conserved within subfamilies are shown on the consensus line in regular type and separated by a gray line. A gray line also separates members of the two SNF1 subfamilies as denoted on the left side. Gaps (−) were introduced to maximize the alignment. Numbering is shown on top and is relative to Hunk spacing. Database accession numbers used are as follows: 80944 (Wpk4); 3089349 (C-TAK1); Z83868 (MARK1); Z83869 (MARK2); U22183 (PAR-1); U11494 (Msk); X89383 (SNRK); JC1446 (AKin10); A56009 (NPK5); S24578 (BKIN12); A41361 (Rkin1); Z29486 (AMPK); A26030 (SNF1); X95351 (Melk); and A38903 (*S. pombe* KIN1).

ogy to other members of the SNF1 family or to other known molecules.

Chromosomal Localization

The mouse chromosomal location of *Hunk* was determined by interspecific backcross analysis using progeny derived from matings of [(C57BL/6J × *M. spretus*)F_1 × C57BL/6J] mice (Fig. 4). This interspecific backcross mapping panel has been typed for over 2800 loci that are well distributed among all the autosomes as well as the X chromosome (Copeland and Jenkins, 1991). C57BL/6J and *M. spretus* DNA samples were digested with several enzymes and analyzed by Southern blot hybridization for informative restriction fragment length polymorphisms (RFLPs) using a mouse *Hunk* cDNA probe. The 5.8-kb *Sac*I *M. spretus* RFLP (see Materials and Methods) was used to follow the segregation of the *Hunk* locus in backcross mice. The mapping results indicated that *Hunk* is located in the distal region of mouse chromosome 16 linked to *App, Tiam1,* and *Erg.* Although 104 mice were analyzed for every marker and are shown in the segregation analysis (Fig. 4), up to 152 mice were typed for some pairs of markers. Each locus was analyzed in pairwise combinations for recombination frequencies using the additional data. The ratios of the total number of mice exhibiting recombinant chromosomes to the total number of mice analyzed for each pair of loci and the most likely gene order are: centromere–*App*–4/123–*Hunk*–0/130–*Tiam1*–4/152–*Erg.* The recombination frequencies (expressed as genetic distances in centimorgans ± the standard error) are –*App*–3.3 ± 1.6 (*Hunk, Tiam1*)–2.6 ± 1.3–*Erg.* No recombinants were detected between *Hunk* and *Tiam1* in 130 animals typed in common, suggesting that the two loci are within 2.3 cM of each other (upper 95% confidence limit).

We have compared our interspecific map of chromosome 16 with a composite mouse linkage map that reports the map location of many uncloned mouse mutations (http://www.informatics.jax.org/). *Hunk* mapped in a region of the composite map that lacks mouse mutations (data not shown).

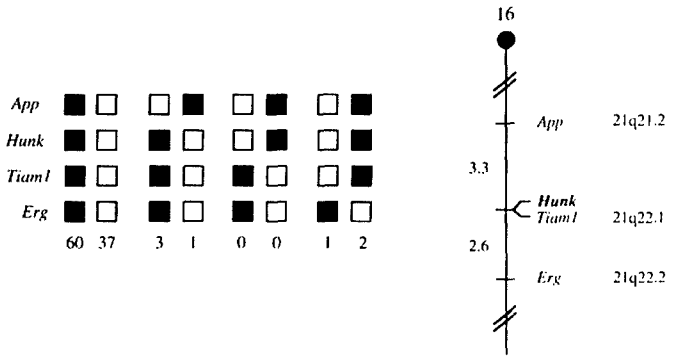

FIG. 4. *Hunk* maps in the distal region of mouse chromosome 16. *Hunk* was mapped to mouse chromosome 16 by interspecific backcross analysis. The segregation patterns of *Hunk* and flanking genes in 104 backcross animals that were typed for all loci are shown at the top of the figure. For individual pairs of loci, more than 104 animals were typed (see text). Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6J × *M. spretus*)F_1 parent. The shaded boxes represent the presence of a C57BL/6J allele, and white boxes represent the presence of a *M. spretus* allele. The number of offspring inheriting each type of chromosome is listed at the bottom of each column. A partial chromosome 16 linkage map showing the location of *Hunk* in relation to linked genes is shown at the right. Recombination distances between loci in centimorgans are shown to the left of the chromosome, and the positions of loci in human chromosomes, where known, are shown to the right. References for the human map positions of loci cited in this study can be obtained from the GDB (Genome Data Base).

Hunk Encodes a Functional Protein Kinase

To address whether *Hunk* encodes a functional kinase, transgenic mice were engineered to overexpress Hunk in the mammary gland using the mouse mammary tumor virus LTR to direct *Hunk* expression (Gardner *et al.*, submitted for publication). Affinity-purified amino-terminal and carboxyl-terminal anti-Hunk antisera were used in immunoblotting experiments to detect Hunk in protein extracts prepared from the mammary glands of wildtype mice or MMTV-*Hunk* transgenic mice harvested at day 9 of lactation (Fig. 5A). Consistent with the degree of overexpression estimated from steady-state mRNA levels (data not shown), substantially higher levels of Hunk were detected in extracts prepared from transgenic compared with wildtype mammary glands. No Hunk protein was detected in extracts prepared from a mammary epithelial cell line previously shown not to express *Hunk* mRNA.

To demonstrate that Hunk protein levels are correlated with kinase activity, *in vitro* kinase assays were performed. Affinity-purified anti-Hunk antisera were used to immunoprecipitate Hunk from protein extracts prepared from the mammary glands of wildtype mice, transgenic mice overexpressing Hunk, or a mammary epithelial cell line that does not express *Hunk* mRNA. The resulting immunoprecipitates were incubated with [γ-^{32}P]ATP and either histone H1 or myelin basic protein as substrates (Fig. 5B; and data not shown). Hunk immunoprecipitates were able to phosphorylate both histone H1 and MBP *in vitro*. As predicted based on the relative quantities of Hunk immunoprecipitated from transgenic and wildtype mammary glands, Hunk-associated phosphotransferase activity was substantially greater in immunoprecipitates prepared from transgenic compared to wildtype mammary glands. No activity was observed in immunoprecipitates prepared from a cell line known not to express *Hunk* mRNA. These findings demonstrate that anti-Hunk antisera coimmunoprecipitate Hunk and a phosphotransferase, strongly suggesting that *Hunk* encodes a functional protein kinase.

Analysis of Hunk mRNA Expression

To begin to analyze the biological role played by *Hunk*, the spatial and temporal pattern of mRNA expression of this gene was determined both during fetal development and in adult tissues in the mouse. Northern hybridization analysis was performed on RNA isolated from FVB embryos at embryonic days E6.5, E13.5, and E18.5 using a unique *Hunk* cDNA probe. *Hunk* expression was not detected at E6.5, was dramatically up-regulated at E13.5, and was subsequently down-regulated at E18.5 (Fig. 6A). Similar to results obtained in mammary epithelial cells, analysis of embryonic mRNA revealed *Hunk* mRNA transcripts approximately 5.1 and 5.6 kb in length. Unlike expression in the mammary epithelial cell line, however, the 5.6-kb Hunk mRNA transcript was more abundant than the 5.1-kb transcript at E13.5, whereas the abundance of the two transcripts was equivalent at E18.5. This pattern suggests that *Hunk* transcripts may be regulated in both a developmental stage-specific and a tissue-specific manner.

To determine the spatial localization of *Hunk* mRNA expression during fetal development, ^{35}S-labeled antisense probes were used to perform *in situ* hybridization on E13.5 and E18.5 embryos (Figs. 6B–6K). These studies revealed intense organ-specific expression of *Hunk* mRNA at E13.5 in the brain, skin, and developing bone, as well as more diffuse expression throughout the embryo. Expression of *Hunk* was more restricted at

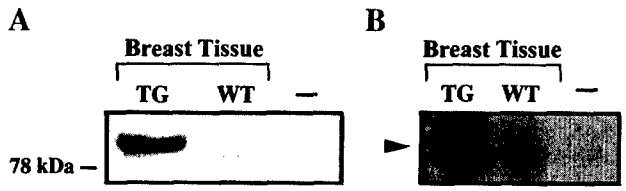

FIG. 5. Kinase activity associated with the *Hunk* gene product. (**A**) Immunoblotting using amino-terminal anti-Hunk antisera to analyze Hunk protein expression. 50 μg of protein extract prepared from mammary glands harvested from either MMTV-Hunk transgenic (TG) or wildtype (WT) mice, or 100 μg of protein extract prepared from HC11 cells, a mammary epithelial cell line that does not express *Hunk* mRNA (–), was analyzed by immunoblotting using amino-terminal anti-Hunk antisera. The relative migration of the 78-kDa marker is indicated. (**B**) *In vitro* kinase assay of Hunk immunoprecipitates. Histone H1 was used as an *in vitro* kinase substrate for Hunk protein immunoprecipitated from extracts containing 205 μg of protein as in Fig. 2B. An arrowhead indicates the relative migration of histone H1.

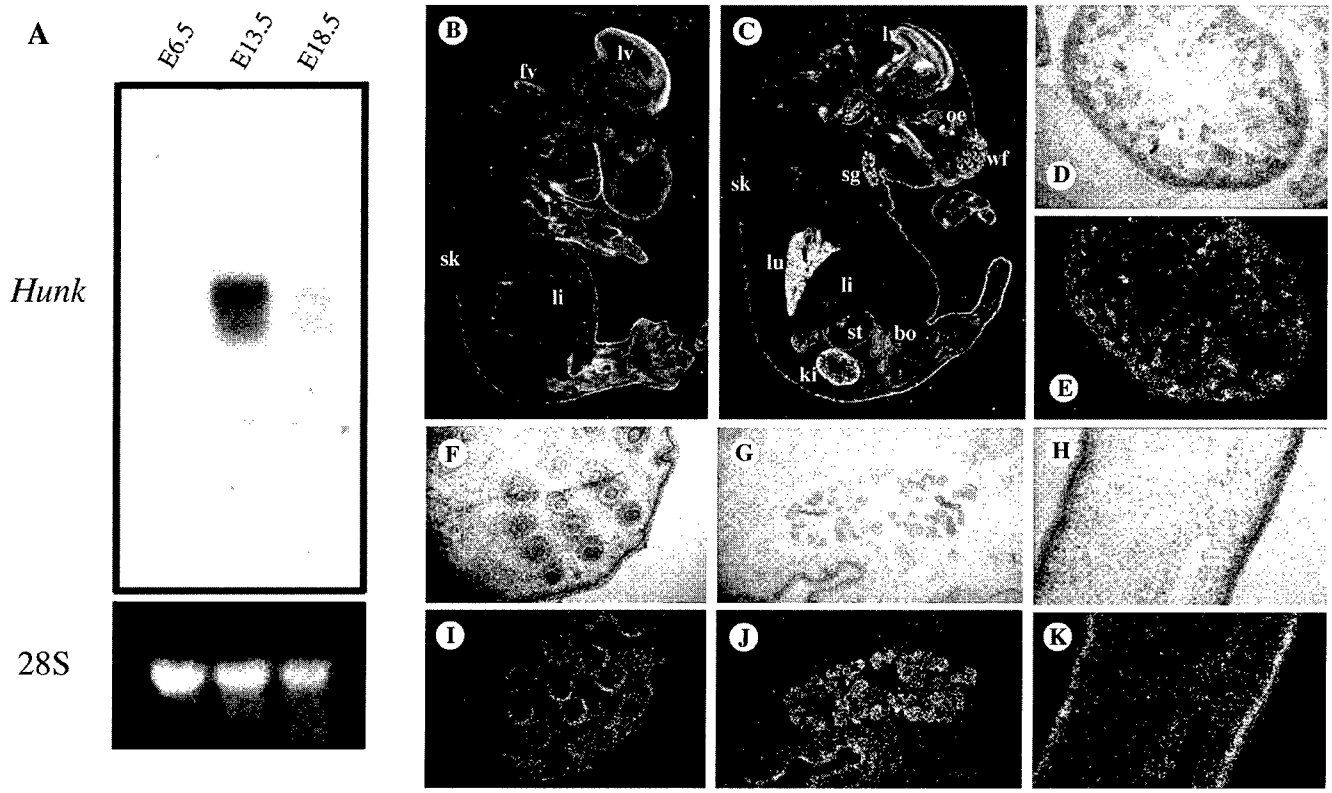

FIG. 6. Expression of *Hunk* during murine embryogenesis. (**A**) Northern hybridization analysis of 2 μg of poly(A)$^+$ RNA from day E6.5, E13.5, and E18.5 embryos hybridized with a cDNA probe specific for *Hunk*. The 28S ribosomal RNA band is shown as a loading control. (**B–K**) *In situ* hybridization analysis of *Hunk* mRNA expression . Bright-field (**D, F, G, H**) and dark-field (**B, C, E, I, J, K**) photomicrographs of E13.5 (**B**) and E18.5 (**C–K**) FVB embryo sections hybridized with an ^{35}S-lableled *Hunk* antisense cDNA probe. Tissues shown are kidney (**D, E**), whisker hair follicles (**F, I**), submandibular gland (**G, J**), and skin (**H, K**). No signal over background was detected in serial sections hybridized with sense *Hunk* probes. bo, bowel; fv, fourth ventricle; ki, kidney; li, liver; lu, lung; lv, lateral ventricle; oe, olfactory epithelium; sg, submandibular gland; sk, skin; st, stomach; wf, whisker hair follicle. Magnification: 8× (**B, C**); 20× (**D–K**). Exposure times were optimized for each panel.

E18.5, with particularly prominent hybridization in the brain, lung, salivary gland, olfactory epithelium, skin, whisker hair follicles, and kidney. Thus, *Hunk* expression during fetal development occurs in a developmentally regulated and tissue-specific manner.

The distribution of *Hunk* expression in tissues of the adult mouse was analyzed by RNase protection (Fig. 7A). High levels of *Hunk* expression were detected in ovary, thymus, lung, and brain, with modest levels of expression in breast, uterus, liver, kidney, and duodenum. *Hunk* mRNA expression was very low or undetectable in heart, skeletal muscle, testis, spleen, and stomach.

The spatial pattern of *Hunk* expression was determined in murine tissues by *in situ* hybridization (Figs. 7B–7M). Interestingly, this analysis revealed that *Hunk* is expressed in only a subset of cells within each expressing organ. In the duodenum, *Hunk* is expressed in a subset of epithelial cells located in duodenal crypts, whereas little or no expression is observed in more differentiated epithelial cells of the duodenum or in the mesenchymal compartment of this tissue (Figs. 7B and 7C). Heterogeneity is also observed among the crypt cells themselves, whereby cells expressing *Hunk* mRNA at high levels are located adjacent to cells expressing *Hunk* at substantially lower levels. Heterogeneous expression patterns are also observed in other tissues. For instance, *Hunk* mRNA expression in the uterus is restricted to isolated epithelial cells located in mesometrial glands (Figs. 7D and 7E). Similarly, *Hunk* expression in the prostate is found within only a subset of ductal epithelial cells (Figs. 7F and 7G). *Hunk* expression in the ovary is found principally in the stroma, with little or no expression detected in developing follicles or corpora lutea (Figs. 7H and 7I). *Hunk* expression in the thymus is limited primarily to the thymic medulla with lower levels of expression in the thymic capsule (Figs. 7J and 7K). High-power examination revealed that, as in other tissues, expression in the thymic medulla is markedly heterogeneous (Fig. 7L). *Hunk* is expressed throughout the brain, with particularly high levels in the cortex, dentate gyrus, and CA1–3 region of the hippocampus (Fig. 7M). High-power examination also revealed marked heterogeneity in *Hunk* expression among different cell types in the cerebral cortex (data not shown). Thus, *Hunk* is expressed in a variety of tissues of the adult mouse, and expression within these tissues is generally restricted to a subset of cells within a particular compartment or compartments.

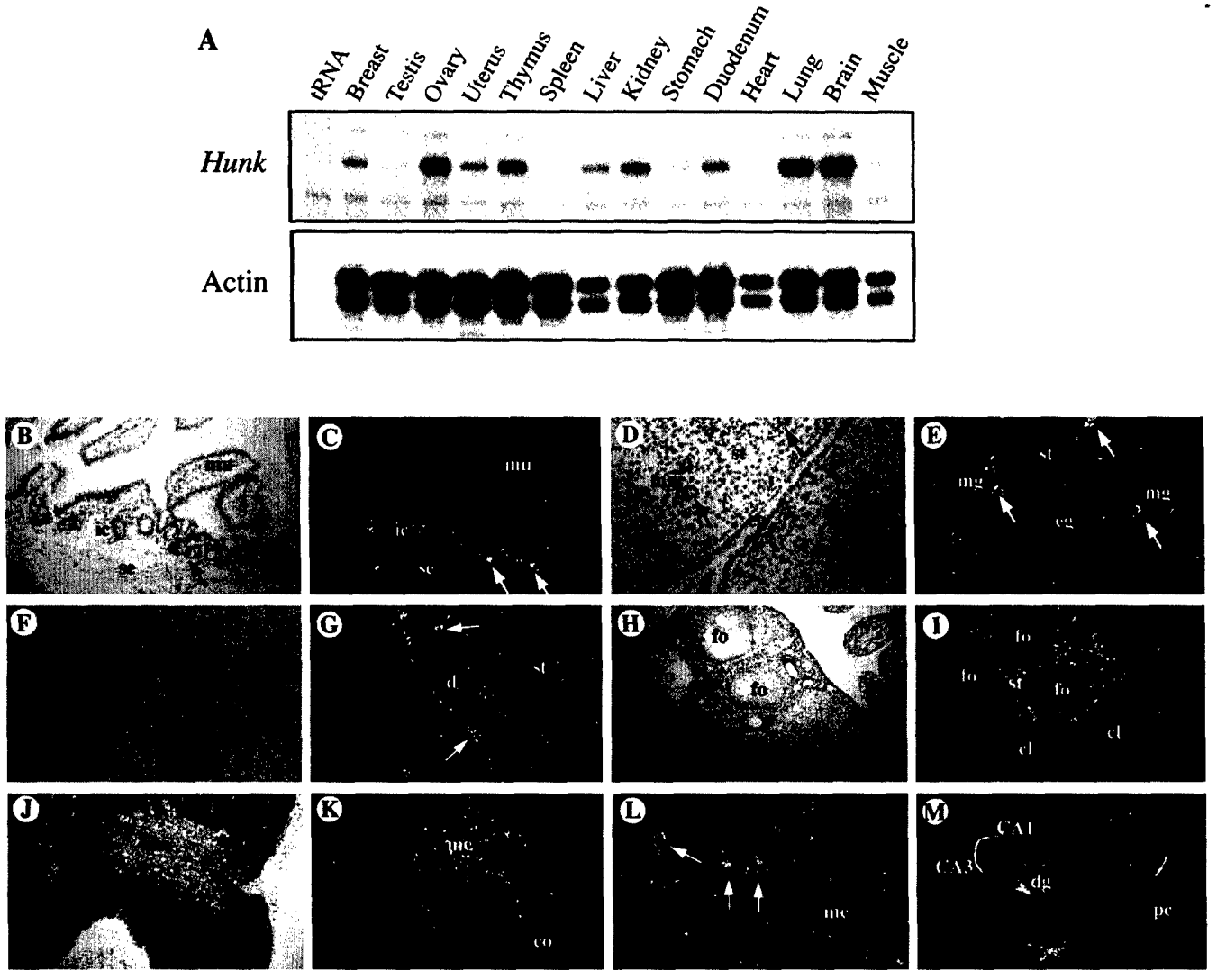

FIG. 7. Tissue-specific expression of *Hunk* in adult tissues. (**A**) RNase protection analysis of *Hunk* mRNA expression in tissues of the adult mouse. 30 μg of RNA isolated from the indicated murine tissues was hybridized with antisense RNA probes specific for *Hunk* and for *β-actin*. (**B–M**) Spatial localization of *Hunk* expression in tissues of the adult mouse. Bright-field (**B, D, F, H, J**) and dark-field (**C, E, G, I, K, L, M**) photomicrographs of *in situ* hybridization analysis performed on sections of duodenum (**B, C**), uterus (**D, E**), prostate (**F, G**), ovary (**H, I**), thymus (**J–L**), and brain (**M**), hybridized with a ^{35}S-labeled *Hunk* antisense probe. No signal over background was detected in serial sections hybridized with a sense *Hunk* probe. Arrows indicate cells expressing *Hunk* at high levels. CA1 and CA3, regions of the hippocampus; cl, corpus luteum; co, cortex; d, epithelial duct; dg, dentate gyrus; eg, endometrial gland; fo, follicle; ic, intestinal crypt; me, medulla; mg, mesometrial gland; mu, mucosa; pc, parietal cortex; se, serosa; st, stroma. Magnification: 10× (**M**), 90× (**H–K**), 180× (**B, C**), 300× (**D, E**) or 500× (**F, G, L, M**).

DISCUSSION

We initially identified the novel serine/threonine kinase, *Hunk,* in a screen designed to isolate protein kinases involved in mammary development and carcinogenesis. We have now described the cloning, chromosomal localization, and activity of this kinase and have characterized its expression in the mouse. *Hunk* is located on distal mouse chromosome 16, is transcribed as 5.1- and 5.6-kb mRNA species, and encodes an 80-kDa protein containing each of the amino acid motifs characteristic of serine/threonine kinases. Consistent with this, antisera that specifically immunoprecipitate Hunk coimmunoprecipitate phosphotransferase activity, and overexpression of Hunk in mammary epithelial cells increases the level of this phosphotransferase activity. *Hunk* expression in the mouse is developmentally regulated and tissue-specific both during fetal development and in the adult. Interestingly, within multiple tissues *Hunk* expression is restricted to subsets of cells within specific cellular compartments. These data suggest a role for Hunk in developmental processes in multiple tissues.

Several lines of evidence suggest that the *Hunk* cDNA sequence obtained represents the full-length *Hunk* ORF. First, Northern hybridization analysis of poly(A)$^+$ RNA isolated from mammary epithelial cell lines using a *Hunk*-specific cDNA probe identifies a predominant mRNA species 5.1 kb in length, consistent with the 5025-nt cDNA sequence obtained for clone E8. Second, *in vitro* transcription and translation

of clone E8 yield a polypeptide that is detected by anti-Hunk antisera, that comigrates with endogenous Hunk, and whose size is consistent with that predicted for the *Hunk* ORF. Third, comparison of the sequence of clone E8 with a recently isolated human *Hunk* cDNA clone reveals a high level of homology within the predicted ORF and a lower level of homology 5′ of the predicted initiation codon and 3′ of the predicted termination codon (H. P. Gardner *et al.,* in preparation). Finally, the observation that anti-Hunk antisera appear to recognize a single polypeptide species in lysates from cells known to express both transcripts provides evidence that we have isolated the entire ORF and that the 5.6-kb *Hunk* mRNA contains additional 5′ or 3′ untranslated sequence. Taken together, these findings suggest that the cDNA clones isolated represent a full-length *Hunk* transcript and contain the complete coding region.

Within the kinase catalytic domain, Hunk is most closely related to the SNF1 family of protein kinases, although Hunk appears to define a new branch on the SNF1 family tree. SNF1 is composed of a heterotrimeric complex that is activated by glucose starvation and is required for the expression of genes in response to nutritional stress (Carlson *et al.,* 1981; Celenza *et al.,* 1989; Ciriacy, 1977; Fields and Song, 1989; Wilson *et al.,* 1996; Yang *et al.,* 1992, 1994; Zimmermann *et al.,* 1977). Like SNF1, the mammalian SNF1-related kinase, AMPK, is involved in the cellular response to environmental stresses, particularly those that elevate cellular AMP:ATP ratios. Once activated, AMPK functions to decrease energy-requiring anabolic pathways such as sterol and fatty acid synthesis while upregulating energy-producing catabolic pathways such as fatty acid oxidation (Moore *et al.,* 1991; Ponticos *et al.,* 1998). AMPK complements the *snf1* mutation in yeast and phosphorylates some of the same targets as SNF1 (Hardie, 1999; Hardie *et al.,* 1997, 1999; Woods *et al.,* 1996). Like SNF1, AMPK is a heterotrimer composed of α, β, and γ subunits that are homologous to the subunits of SNF1 (Hardie, 1999). Thus, AMPK and SNF1 are closely related both functionally and structurally, demonstrating that the regulatory pathways in which they operate have been highly conserved during evolution.

Other SNF1 family members in plants, including Rkin1, BKIN12, AKin10, NPK5, and Wpk4, have been implicated in nutritional and environmental stress responses (Alderson *et al.,* 1991; Muranaka *et al.,* 1994; Sano and Youssefian, 1994; Wilson *et al.,* 1996). Like Hunk, several plant SNF1 family members are expressed in a tissue-specific manner. For example, AKIN10 is expressed in roots, shoots, and leaves, whereas RKIN1 is detected in developing endosperms but not in shoots (Alderson *et al.,* 1991; Le Guen *et al.,* 1992).

More recently, SNF1-related kinases have been identified in mammals and have been implicated in development processes, particularly in the regulation of cellular proliferation and differentiation. For instance, C-TAK1/p78 appears to be involved in cell cycle regulation based on its ability to phosphorylate and inactivate Cdc25c (Peng *et al.,* 1997, 1998). Since Cdc25c controls entry into mitosis by activating cdc2, inactivation of Cdc25c by C-TAK1 would be predicted to regulate proliferation negatively. Consistent with this model, C-TAK1/p78 is down-regulated in adenocarcinomas of the pancreas (Parsa, 1988).

Perhaps the most compelling evidence that SNF1 kinases are involved in development is the observation that mutations in the *C. elegans* SNF1-related kinase PAR-1 result in an inability to establish polarity in the developing embryo (Guo and Kemphues, 1995). Specifically, *par-1* mutations disrupt P granule localization, asymmetric cell divisions, blastomere fates, and mitotic spindle orientation during early embryogenesis. In an analogous manner, the mammalian PAR-1 homologue MARK2/Emk is asymmetrically localized in epithelial cells in vertebrates, and expression of a dominant negative form of MARK2 disrupts both cell polarity and epithelial cell–cell contacts (Bohm *et al.,* 1997). In addition, overexpression of either MARK2 or its close family member MARK1 results in hyperphosphorylation of microtubule-associated proteins, disruption of the microtubule array, and cell death (Drewes *et al.,* 1997).

Additional SNF1-related molecules, such as Msk and SNRK, have been implicated in vertebrate differentiation and development on the basis of their temporal and spatial patterns of expression (Becker *et al.,* 1996; Ruiz *et al.,* 1994). For example, *Msk* is expressed in presumptive myocardial cells during embryogenesis and is down-regulated following primitive heart tube formation, whereas SNRK is up-regulated during adipocyte differentiation. In a similar manner, our analysis of *Hunk* mRNA expression patterns suggests the possibility of a developmental role for Hunk in specific tissues. *Hunk* is expressed at high levels in the embryo during midgestation as cells are rapidly proliferating and differentiating and is down-regulated in the embryo prior to parturition. During fetal development, *Hunk* mRNA is expressed in a tissue-specific manner and is restricted to particular compartments within expressing tissues. Similarly, *Hunk* is also expressed in a tissue-specific manner in the adult mouse, and its expression is restricted to subsets of cells within these tissues. In aggregate, these data indicate that SNF1 family members participate in a wide range of developmental processes in higher eukaryotes and suggest that Hunk may also play an important role in one or more of these processes.

Outside the catalytic domain, a region of homology exists between SNF1 family members previously described as the SNH, or SNF1 homology region (Becker *et al.,* 1996). Since the distance between the catalytic domain and the SNH is conserved and since many kinases contain autoregulatory domains, it is plausible that the SNH domain functions to regulate kinase ac-

tivity (Yokokura *et al.,* 1995). Consistent with this speculation is the presence of weak homology between the SNH domain of SNF1 kinases and the autoinhibitory domain of the closely related family of calcium–calmodulin regulated kinases (data not shown). This homology does not extend into the adjacent calmodulin binding region, consistent with the observation that SNF1 kinases are not regulated by calcium. Regardless, the presence of the SNH domain in all SNF1 kinases raises the possibility that members of this family of molecules may be regulated by a common mechanism.

The distal portion of mouse chromosome 16 shares a region of conserved synteny with human chromosome 21q (summarized in Fig. 4). In particular, *Tiam1* has been mapped to 21q22.1. Mutations or segmental trisomy in this region of human chromosome 21 are associated with Alzheimer disease and Down syndrome, respectively. The close linkage between *Tiam1* and *Hunk* in the mouse suggests that the human homologue of *Hunk* will map to 21q22, as well. In fact, BLAST alignment of *Hunk* to sequences in GenBank reveals homology to human genomic DNA sequences cloned from 21q22.1 (gi4835629). This indicates that the human homologue of *Hunk* lies within a region of chromosome 21q22 believed to contribute to several of the phenotypic features characteristic of Down syndrome (Delabar *et al.,* 1993; Korenberg *et al.,* 1994; Rahmani *et al.,* 1989). In this regard, it is interesting to note that *Hunk* is expressed at high levels throughout the brain during murine fetal development as well as in the adult, with particularly high levels being found in the hippocampus, dentate gyrus, and cortex. Whether increased Hunk expression in the brain is related to the pathophysiology of Alzheimer disease or Down syndrome is unknown.

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Cloning, Characterization, and Chromosomal Localization of *Pnck*, a Ca^{2+}/Calmodulin-Dependent Protein Kinase

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Calcium is an important second messenger in eukaryotic cells. Many of the effects of calcium are mediated via its interaction with calmodulin and the subsequent activation of Ca^{2+}/calmodulin-dependent (CaM) kinases. CaM kinases are involved in a wide variety of cellular processes including muscle contraction, neurotransmitter release, cell cycle control, and transcriptional regulation. While CaMKII has been implicated in learning and memory, the biological role of the other multifunctional CaM kinases, CaMKI and CaMKIV, is largely unknown. In the course of a degenerate RT-PCR protein kinase screen, we identified a novel serine/threonine kinase, *Pnck*. In this report, we describe the cloning, chromosomal localization, and expression of *Pnck*, which encodes a 38-kDa protein kinase whose catalytic domain shares 45–70% identity with members of the CaM kinase family. The gene for *Pnck* localizes to mouse chromosome X, in a region of conserved synteny with human chromosome Xq28 that is associated with multiple distinct mental retardation syndromes. *Pnck* is upregulated during intermediate and late stages of murine fetal development with highest levels of expression in developing brain, bone, and gut. *Pnck* is also expressed in a tissue-specific manner in adult mice with highest levels of expression detected in brain, uterus, ovary, and testis. Interestingly, *Pnck* expression in these tissues is restricted to particular compartments and appears to be further restricted to subsets of cells within those compartments. The chromosomal localization of *Pnck*, along with its tissue-specific and restricted pattern of spatial expression during development, suggests that *Pnck* may be involved in a variety of developmental processes including development of the central nervous system.

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession No. AF181984.

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INTRODUCTION

Many protein kinases function as intermediates in signal transduction pathways that control complex processes such as differentiation, development, and carcinogenesis (Birchmeier *et al.*, 1993; Bolen *et al.*, 1992; Rawlings and Witte, 1994). Accordingly, studies of protein kinases in a wide range of biological systems have led to a more comprehensive understanding of the regulation of cell growth and differentiation (Bolen, 1993; Fantl *et al.*, 1993; Hardie, 1990). Not surprisingly, several members of the protein kinase family have been shown to be involved in the pathogenesis of cancer both in humans and in rodent model systems (Cardiff and Muller, 1993; Dickson *et al.*, 1992; Guy *et al.*, 1992, 1994; Slamon *et al.*, 1989). In light of these findings, we performed a screen designed to identify and study the role of protein kinases in mammary development and carcinogenesis (Chodosh *et al.*, 2000, in press; Gardner *et al.*, in press; Stairs *et al.*, 1998). In the course of these studies, we identified a novel serine/threonine kinase, *Pnck* (pregnancy-upregulated nonubiquitous CaM kinase), that is related to the Ca^{2+}/calmodulin-dependent (CaM) family of protein kinases.

Ca^{2+} is an important intracellular second-messenger molecule in eukaryotic signal transduction pathways. Many of the effects of Ca^{2+} are mediated through its interaction with the Ca^{2+}-binding protein, calmodulin. The Ca^{2+}/calmodulin complex is, in turn, required for maximal activation of CaM-dependent protein kinases, which ultimately regulate cellular processes as diverse as neurotransmitter release, metabolism, and gene transcription (Fukunaga and Miyamoto, 1999; Lukas *et al.*, 1998; Matthews *et al.*, 1994; Nairn and Piciotto, 1994; Polishchuk *et al.*, 1995; Schulman, 1993; Sheng *et al.*, 1991). In addition to their regulation by CaM, CaM kinases share structural and functional homology both in the kinase catalytic domain and in a regulatory region composed of composite autoinhibitory and CaM-

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binding domains (Hanks and Quinn, 1991; Hanks *et al.,* 1988; Haribabu *et al.,* 1995; Knighton *et al.,* 1992; Picciotto *et al.,* 1996; Yokokura *et al.,* 1995).

Despite these similarities, significant differences exist between CaM kinase family members. For instance, this family includes members with high substrate specificity, such as myosin light-chain kinase (MLCK) and phosphorylase kinase, as well as members with broader substrate specificities collectively referred to as the multifunctional CaM kinases, such as CaMKI, CaMKIV, and members of the CaMKII subfamily (Braun and Schulman, 1995; Cawley *et al.,* 1993; Herring *et al.,* 1990; Matthews *et al.,* 1994; Schulman, 1993). Other properties that differ among CaM kinase family members include their subcellular localization, regulation by autophosphorylation, and regulation by other proteins. In addition, CaM kinases have unique amino- and carboxyl-terminal domains that contribute to kinase-specific differences in subcellular localization, subunit interactions, and other protein–protein interactions.

Much of the information available regarding the multifunctional CaM kinases is derived from studies conducted in the brain, in part because each of these kinases is expressed at high levels in this organ. However, CaMKII is the only multifunctional CaM kinase whose biological function has been defined (Hanley *et al.,* 1987; Jensen *et al.,* 1991b; Lin *et al.,* 1987; Picciotto *et al.,* 1993; Tobimatsu and Fujisawa, 1989; Tobimatsu *et al.,* 1988). The CaMKII holoenzyme is an oligomeric complex composed of combinations of independently encoded, highly homologous α, β, γ, and δ subunits. Mice with targeted disruption of CaMKIIα are deficient in long-term potentiation and exhibit specific defects in learning and memory (Silva *et al.,* 1992a, b). Unlike the α subunit of CaMKII whose expression is restricted to the brain, CaMKI, CaMKIV, and the δ and γ subunits of CaMKII have a broader tissue distribution and therefore presumably have as yet unrecognized functions in other tissues (Hanley *et al.,* 1987; Lin *et al.,* 1987; Naito *et al.,* 1997; Picciotto *et al.,* 1993, 1995; Tobimatsu and Fujisawa, 1989; Tobimatsu *et al.,* 1988).

In this report, we describe the cloning, chromosomal localization, and initial characterization of *Pnck,* a member of the CaM kinase family of protein serine/threonine kinases. We have isolated cDNA clones for *Pnck* that encode a 38-kDa polypeptide. The gene for *Pnck* localizes to mouse chromosome X in a region of conserved synteny with human chromosome Xq28 that has been implicated in distinct mental retardation syndromes (Lubs *et al.,* 1999). *Pnck* expression is developmentally regulated and tissue-specific during murine fetal development with high levels of expression in developing brain, bone, and gut. *Pnck* expression is also tissue-specific in adult mice with highest levels of expression in the hippocampus and dentate gyrus of the brain. Interestingly, within expressing tissues, *Pnck* expression is restricted to subsets of cells within particular compartments. These data suggest a role for *Pnck* in the development of the central nervous system and other tissues.

MATERIALS AND METHODS

Cloning of a full-length Pnck cDNA. The original catalytic domain fragment, *Bstk3,* was isolated from first-strand cDNA derived from mammary glands of mice at day 2 of postlactational involution using the degenerate oligonucleotide primers BSTKIa (5′-GGGCCCGGATCC(G/A)T(A/G)CAC(A/C)G(A/G/C)GAC(C/T)T-3′) and PTKIIa (5′-CCCGGGGAATTCCA(A/T)AGGACCA(G/C)AC(G/A)TC-3′) (Chodosh *et al.,* in press; Wilks, 1989, 1991; Wilks *et al.,* 1989). This original fragment, corresponding to nucleotides 501 to 704 of full-length *Pnck,* was used to screen 5×10^5 lambda phage plaques from an oligo(dT)-primed murine brain cDNA library according to standard protocols (CPMB). Primary screening yielded a total of 73 clones of varying hybridization intensity that were positive on duplicate filters. Ten clones with medium to high hybridization intensity were plaque purified, and plasmids were liberated by *in vivo* excision according to the manufacturer's instructions (Stratagene). Sequence analysis of 5 of these clones revealed a high level of homology to CaMKI. The remaining 5 clones were found to encode portions of *Pnck* as determined by overlapping sequence identity to one another and to *Bstk3.* Two clones were not studied further since one clone was chimeric and a second clone contained only partial *Pnck* sequence. Three nonchimeric clones, U7, V1, and Q3, were completely sequenced by automated sequencing using an ABI Prism 377 DNA sequencer. Nucleotide sequence alignment revealed no differences between the three clones outside of their respective 5′-untranslated region (UTR) sequences. The full-length *Pnck* cDNA sequence corresponding to the clone with the longest 5′-UTR, U7, has been deposited with the GenBank database (Accession No. AF181984).

Sequence analysis. Sequence analysis, including prediction of open reading frames, calculation of predicted molecular weights, multiple sequence alignment, and phylogenetic analysis, was performed using MacVector (Oxford Molecular Group), ClustalW, ClustalX, and DendroMaker 4.0. Pairwise and multiple sequence alignments of kinase catalytic domains I–XI were performed using the ClustalW alignment program. Calculations were made using the BLOSUM series with an open gap penalty of 10, an extended gap penalty of 0.05, and a delay divergent of 40%. Phylogenetic calculations with the same parameters were performed using the ClustalX multisequence alignment program. An unrooted phylogenetic tree was drawn using DendroMaker 4.0.

Tissue preparation. FVB mouse embryos were harvested at specified time points following timed matings. Day 0.5 postcoital was defined as noon of the day on which a vaginal plug was observed. Tissues used for RNA preparation were harvested from 15- to 16-week-old virgin mice and snap-frozen on dry ice. Tissues used for *in situ* hybridization analysis were embedded in OCT compound.

RNA analysis. RNA was prepared by homogenization of snap-frozen tissue samples in guanidinium isothiocyanate supplemented with 7 μl/ml 2-mercaptoethanol followed by ultracentrifugation through cesium chloride as previously described (Marquis *et al.,* 1995; Rajan *et al.,* 1997). Poly(A)$^+$ RNA was selected using oligo(dT) cellulose (Pharmacia), separated on a 1% LE agarose gel, and passively transferred to a Gene Screen membrane (NEN). Northern hybridization was performed as described using a ^{32}P-labeled cDNA probe encompassing nucleotides 1135 to 1509 of *Pnck* generated by random-primed labeling (BMB) (Marquis *et al.,* 1995).

Southern hybridization analysis was performed on a zoo-blot (Clontech) hybridized with a ^{32}P-labeled cDNA probe corresponding to nucleotides 1321 to 1509 from the 3′-UTR of *Pnck.* Hybridization and washes were performed according to the manufacturer's directions (Clontech). A single band was detected in genomic DNA from both mouse and rat, confirming that, under these conditions, this *Pnck*-specific 3′-UTR probe recognizes a single locus.

Ribonuclease protection analysis was performed as described (Marquis *et al.,* 1995). Body-labeled antisense riboprobes were generated using linearized plasmids containing nucleotides 1321 to 1509 of *Pnck* and 1142 to 1241 of *β-actin* (GenBank Accession No. X03672) using [α-^{32}P]UTP and the Promega *in vitro* transcription system with T7 polymerase. A β-actin antisense riboprobe was added to each reaction as an internal control. Probes were hybridized with RNA samples at 58°C overnight in 50% formamide/100 mM Pipes (pH 6.7). Hybridized samples were digested with RNase A and T1, purified, electrophoresed on a 6% denaturing polyacrylamide gel, and subjected to autoradiography.

In vitro transcription and translation. *In vitro* transcription and translation were performed on 1 μg of plasmid DNA using rabbit reticulocyte lysates in the presence of [^{35}S]methionine according to the manufacturer's instructions (TNT kit, Promega). Completed reactions were electrophoresed on a 10% SDS–PAGE gel and were subjected to autoradiography.

Interspecific mouse backcross mapping. Interspecific backcross progeny were generated by mating (C57BL/6J × *M. spretus*)F_1 females and C57BL/6J males as described (Copeland and Jenkins, 1991). A total of 205 N_2 mice were used to map the *Pnck* locus (see text for details). DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer, and hybridization were performed essentially as described (Jenkins *et al.,* 1982). All blots were prepared with Hybond-N$^+$ nylon membrane (Amersham). The probe, a 375-bp fragment corresponding to nucleotides 1135 to 1509 of mouse *Pnck* cDNA, was labeled with [α-^{32}P]dCTP using a nick-translation labeling kit (Boehringer Mannheim); washing was performed at a final stringency of 1.0× SSCP, 0.1% SDS at 65°C. A fragment of 13.0 kb was detected in *Pst*I-digested C57BL/6J DNA, and a fragment of 5.1 kb was detected in *Pst*I-digested *M. spretus* DNA. The presence or absence of the 5.1-kb *Pst*I *M. spretus*-specific fragment was followed in backcross mice.

A description of the probes and RFLPs for the loci linked to *Pnck* including *Tnfsf5, Il1rak,* and *Ar* has been reported previously (Centanni *et al.,* 1998). Recombination distances were calculated using Map Manager, version 2.6.5. Gene order was determined by minimizing the number of recombination events required to account for the allele distribution patterns.

In situ hybridization. *In situ* hybridization was performed as described (Marquis *et al.,* 1995). Antisense and sense probes were synthesized with the Promega *in vitro* transcription system using ^{35}S-UTP and ^{35}S-CTP from the T7 and SP6 RNA polymerase promoters of a PCR template containing sequences corresponding to nucleotides 1135 to 1509 of *Pnck.*

RESULTS

To isolate regulatory molecules potentially involved in development and carcinogenesis, a degenerate RT-PCR-based screen was performed to identify protein kinases expressed in murine breast cancer cell lines and in the mammary gland during development (Chodosh *et al.,* 1999, in press; Gardner *et al.,* in press; Stairs *et al.,* 1998). This screen resulted in the identification of 41 protein kinases including 33 tyrosine kinases and 8 serine/threonine kinases, 3 of which were novel.

One of these novel kinases, originally called *Bstk3,* is the subject of this study. *Bstk3* was subsequently found to be upregulated in the mammary gland during pregnancy and to be expressed in a punctate pattern in multiple tissues. Therefore, *Bstk3* was renamed *Pnck,* for pregnancy-upregulated, nonubiquitous CaM kinase, to reflect this unique temporal and spatial expression pattern.

Isolation of cDNA Clones Encoding Pnck

Of approximately 1500 clones examined in the context of a screen for expressed protein kinases, a single clone corresponding to *Bstk3* was isolated from the mammary glands of mice undergoing early postlactational regression. To isolate the full-length mRNA transcript from which *Bstk3* was derived, this initial 204-bp RT-PCR product was used to screen a murine brain cDNA library. Three cDNA clones ranging from 1455 to 1554 nucleotides in length were isolated by these means. All three clones were completely sequenced and were found to differ only in their respective 5′-UTRs (Fig. 1B). The sequence of each cDNA clone contains the entire 204-bp RT-PCR fragment, *Bstk3,* as well as a 1029-nucleotide open reading frame (ORF) and a 420-bp 3′-UTR possessing a polyadenylation signal and poly(A) tract (Fig. 1A).

Inspection of the nucleotide sequence surrounding the putative initiation codon at nucleotide 105 of the longest clone, U7, reveals matches with the Kozak translational initiation consensus sequence at positions −1, −3, −5, and −6 (Kozak, 1987, 1991). Conceptual translation of the *Pnck* ORF yields a 343-amino-acid polypeptide of predicted molecular mass 38.6 kDa. The coding sequence for Pnck can be divided into a 14-amino-acid unique amino-terminal segment, a 256-amino-acid kinase catalytic domain, a 41-amino-acid regulatory domain, and a 32-amino-acid unique carboxyl-terminal region. The Pnck kinase catalytic domain contains all of the amino acid motifs conserved among serine/threonine kinases.

To determine whether the lengths of the cDNA clones encoding *Pnck* are consistent with the size of the *Pnck* mRNA transcript, Northern hybridization was performed. Due to potential cross-hybridization between *Pnck* and homologous CaM kinase family members, Southern hybridization was used to confirm the specificity of a probe generated from the 3′-UTR of *Pnck* (data not shown). This *Pnck*-specific probe was used for Northern hybridization analysis performed on poly(A)$^+$ RNA isolated from adult murine brain. Consistent with the lengths of the isolated *Pnck* cDNA clones, this analysis revealed an mRNA transcript approximately 1.6 kb in length (Fig. 2A).

To confirm the coding potential of the *Pnck* ORF, *in vitro* transcription and translation were performed in the presence of [^{35}S]methionine using each of the three *Pnck* cDNA clones as template. In each case, incubation of plasmid DNA with reticulocyte lysate yielded a single labeled polypeptide species of approximately 38 kDa, consistent with the predicted *Pnck* ORF (Fig. 2B). This demonstrates that the predicted initiation codon is capable of functioning as a translation initiation site. Since clone U7 contains multiple in-frame termination codons upstream of this putative initiation codon, these findings suggest that we have isolated the entire *Pnck* coding sequence. However, since the alternate 5′-UTR sequence present in clone V1 does not contain

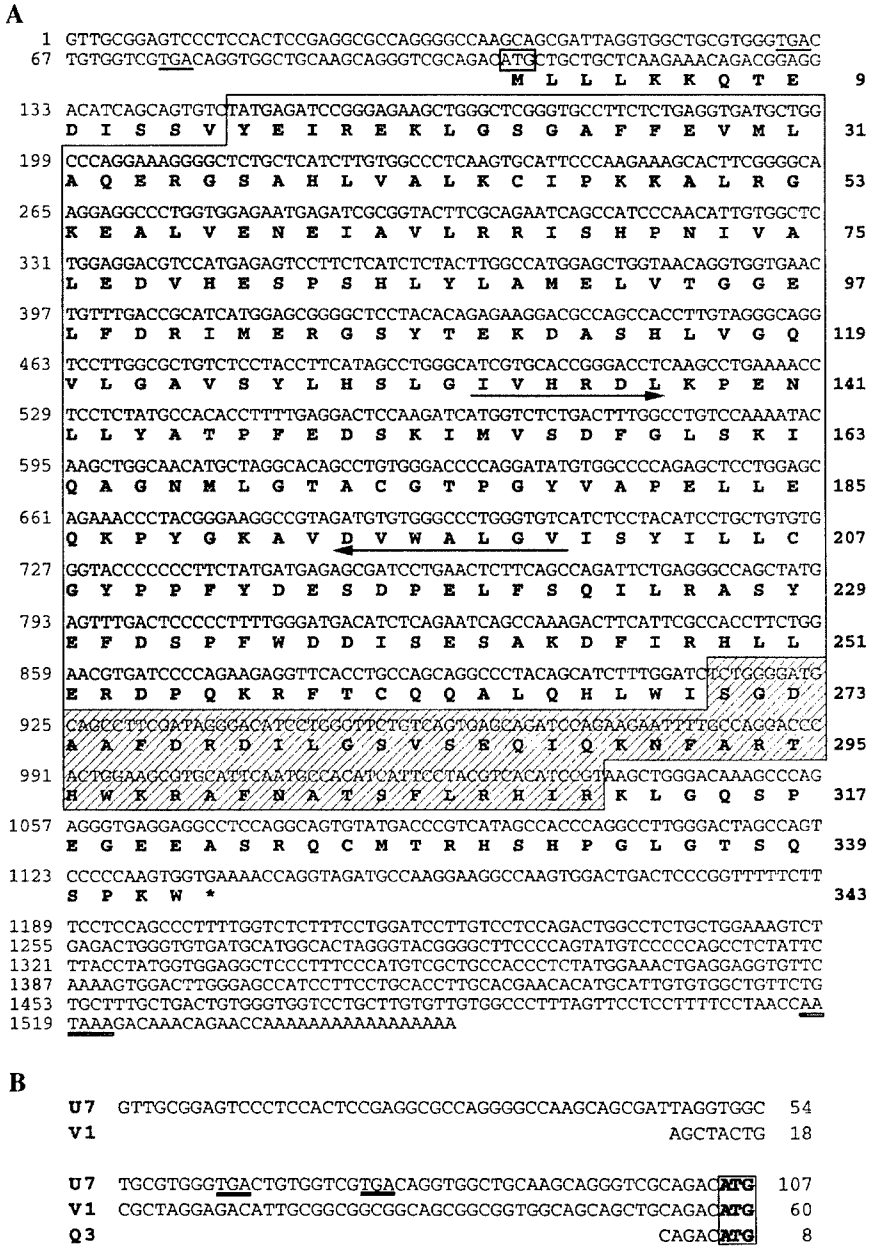

FIG. 1. Nucleotide and deduced amino acid sequence of *Pnck*. (**A**) Composite nucleic acid sequence and conceptual translation of full-length *Pnck* cDNA. Nucleotide coordinates are shown on the left. Amino acid coordinates are shown in boldface type on the right. A shaded box indicates the kinase catalytic domain, and a hatched box denotes the putative regulatory region. The in-frame upstream termination codons in the 5′-UTR and the putative polyadenylation sequence in the 3′-UTR are underlined by thin and thick lines, respectively. The putative initiation codon is boxed, and an asterisk denotes the stop codon. Arrows underline the regions corresponding to the degenerate oligonucleotides used to clone *Bstk3* initially. (**B**) 5′-UTR sequences of three full-length cDNA clones encoding *Pnck*. Nucleotide coordinates relative to each clone are shown to the right. Upstream in-frame termination codons are underlined, and the putative initiation codons are boxed.

an upstream termination codon, we cannot exclude the possibility that alternate polypeptides encoded by the *Pnck* locus that have distinct amino-terminal sequences exist.

Homology to Related Protein Kinases

Multiple sequence alignment of Pnck kinase catalytic subdomains I–XI was used to determine the relationship between Pnck and other CaM kinases (Fig. 3) (Hanks and Quinn, 1991; Hanks *et al.*, 1988). Pnck lies within the group of multifunctional CaM kinases and is most similar to CaMKI. Within the kinase domain, Pnck is 70% identical to CaMKI, 50% identical to CaMKIV, and approximately 45% identical to members of the CaMKII subfamily at the amino acid level. Pnck is also homologous to members of the CaM kinase family in the regulatory domain, although the extent of similarity is lower than that found in the catalytic domain. While Pnck is most closely related to CaMKI in both the catalytic and

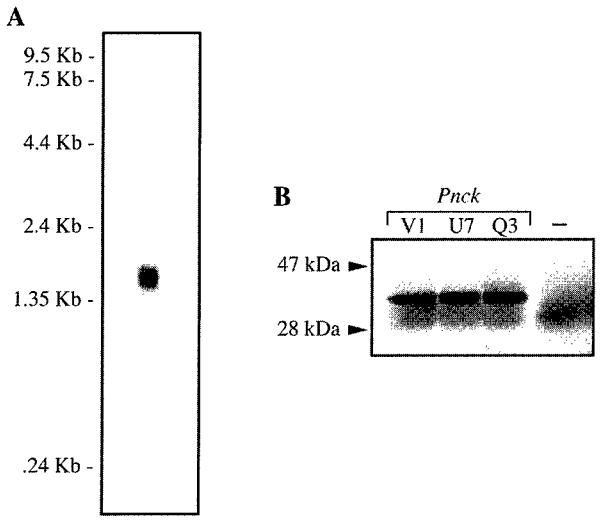

FIG. 2. Expression and coding potential of *Pnck*. (**A**) Northern hybridization analysis of 4 μg poly(A)$^+$ RNA isolated from adult murine brain hybridized with a 3′-UTR probe specific for *Pnck*. The relative migration of RNA size markers is indicated. (**B**) *In vitro* transcription/translation reactions performed using rabbit reticulocyte lysates in the presence of ^{35}S-labeled methionine and 1 μg of template consisting of a full-length *Pnck* cDNA clone (V1, U7, or Q3) or a cDNA plasmid encoding an unrelated kinase (−) as a negative control. IVT reactions were resolved on a 10% SDS–PAGE gel and subjected to autoradiography. The relative migration of molecular weight markers is indicated.

the regulatory domains, the amino acid identity between Pnck and CaMKI is significantly lower than that between CaMKII subfamily members, most of which exhibit greater than 90% amino acid identity within their catalytic and regulatory domains. Moreover, outside of these conserved functional domains, the amino- and carboxyl-terminal regions of Pnck bear no significant similarity to CaMKI, CaMKIV, or other CaM kinase family members.

Chromosomal Localization

The chromosomal location of murine *Pnck* was determined by interspecific backcross analysis using progeny derived from matings of [(C57BL/6J × *Mus spretus*)F_1 ×

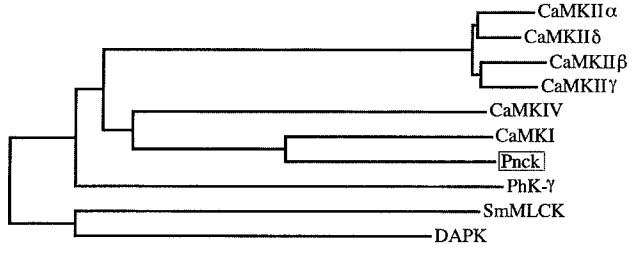

FIG. 3. Homology between Pnck and CaM kinase family members. Phylogenetic tree illustrating the relationship of Pnck kinase catalytic subdomains I–XI to other CaM kinase family members. Analysis and depiction of results were performed using the ClustalX multisequence alignment program and DendroMaker 4.0. Evolutionary relationships are proportional to horizontal branch distances. Database accession numbers used are A30355 (CaMKIIα); A34366 (CaMKIIδ); A26464 (CaMKIIβ); A31908 (CaMKIIγ); M64757 (CaMKIV); L26288 (CaMKI); X07320 (PhK-γ); A41674 (SmMLCK); and X76104 (DAPK).

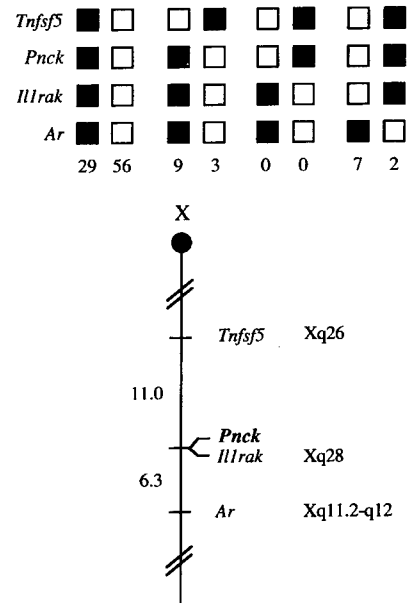

FIG. 4. *Pnck* maps in the central region of the mouse X chromosome. *Pnck* was placed on the mouse X chromosome by interspecific backcross analysis. The segregation patterns of *Pnck* and flanking genes in 106 backcross animals that were typed for all loci are shown at the top of the figure. For individual pairs of loci, more than 106 animals were typed (see text). Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6J × *M. spretus*)F_1 parent. The shaded boxes represent the presence of a C57BL/6J allele, and white boxes represent the presence of a *M. spretus* allele. The number of offspring inheriting each type of chromosome is listed at the bottom of each column. A partial X chromosome linkage map showing the location of *Pnck* in relation to linked genes is shown at the bottom of the figure. Recombination distances between loci in centimorgans are shown to the left of the chromosome, and the positions of loci in human chromosomes are shown to the right. References for the human map positions of loci cited in this study can be obtained from GDB (Genome Data Base).

C57BL/6J] mice. This interspecific backcross mapping panel has been typed for over 2800 loci that are well distributed among all the autosomes as well as the X chromosome (Copeland and Jenkins, 1991). C57BL/6J and *M. spretus* DNAs were digested with several enzymes and analyzed by Southern blot hybridization for informative restriction fragment length polymorphisms (RFLPs) using a cDNA probe specific for the 3′-UTR of *Pnck*. The 5.1-kb *Pst*I *M. spretus* RFLP (see Materials and Methods) was used to follow the segregation of the *Pnck* locus in backcross mice. The mapping results indicated that *Pnck* is located in the central region of the mouse X chromosome linked to *Tnfsf5, Il1rak,* and *Ar*. Although 106 mice were analyzed for every marker and are shown in the segregation analysis (Fig. 4), up to 142 mice were typed for some pairs of markers. Each locus was analyzed in pairwise combinations for recombination frequencies using the additional data. The ratios of the total number of mice exhibiting recombinant chromosomes to the total number of mice analyzed for each pair of loci and the most likely gene order are centromere–*Tnfsf5*–15/137–*Pnck*–0/134–*Il1rak*–9/142–*Ar*. The recombination frequencies expressed as genetic distances in centimorgans ± the standard error are –*Tnfsf5*–11.0 ±

2.7–(*Pnck, Il1rak*)–6.3 ± 2.0–*Ar*. No recombinants were detected between *Pnck* and *Il1rak* in 134 animals typed in common, suggesting that the two loci are within 2.2 cM of each other (upper 95% confidence limit).

We have compared our interspecific map of the X chromosome with a composite mouse linkage map that reports the map location of many uncloned mouse mutations (provided from Mouse Genome Database, http://www.informatics.jax.org/). *Pnck* maps to a region of the composite map that lacks uncloned mouse mutations (data not shown).

The central region of the mouse X chromosome shares a region of conserved homology with the long arm of the human X chromosome (summarized in Fig. 4). In particular, *Il1rak* has been mapped to Xq28. The close linkage between *Il1rak* and *Pnck* in mouse suggests that the human homologue of *Pnck* will map to Xq28, as well.

Analysis of Pnck mRNA Expression

To begin to determine the biological role of *Pnck*, the developmental expression pattern of *Pnck* mRNA was analyzed during murine embryogenesis. Northern hybridization analysis was performed on poly(A)$^+$ RNA isolated from embryos during early, mid-, and late gestation using a *Pnck*-specific probe (Fig. 5A). Compared to mRNA expression levels in early embryogenesis, steady-state *Pnck* mRNA levels are markedly upregulated in the embryo during midgestation and remain elevated through embryonic day 18.5.

To investigate the spatial pattern of *Pnck* expression during fetal development, *in situ* hybridization analysis was performed on embryonic sections at day 14.5 of gestation using an ^{35}S-labeled *Pnck*-specific antisense probe (Fig. 5B). This analysis revealed tissue-specific expression of *Pnck* in the embryo at midgestation with highest levels of expression detected in developing bone, the outer lining of the stomach, and the developing central nervous system, including periventricular regions and the trigeminal ganglion.

The expression profile of *Pnck* in tissues of the adult mouse was determined by RNase protection analysis (Fig. 6A). As in the embryo, *Pnck* expression in the adult mouse is highest in brain. In addition, moderate to low levels of *Pnck* expression are detected in hormonally responsive tissues such as uterus, ovary, testis, and mammary gland, as well as in other tissues such as stomach, heart, and skeletal muscle. Lower, but detectable, levels of *Pnck* expression were observed in thymus, spleen, duodenum, and lung.

Finally, the spatial expression pattern of *Pnck* in adult murine tissues was determined by *in situ* hybridization analysis (Fig. 6B–M). Interestingly, within expressing tissues *Pnck* mRNA was detected in only a subset of cells. In the brain, *Pnck* expression is highest in the dentate gyrus and CA1–3 regions of the hippocampus (Figs. 6B, 6C, 6F, and 6G). *Pnck* is also expressed at relatively high levels in the cortex and is markedly

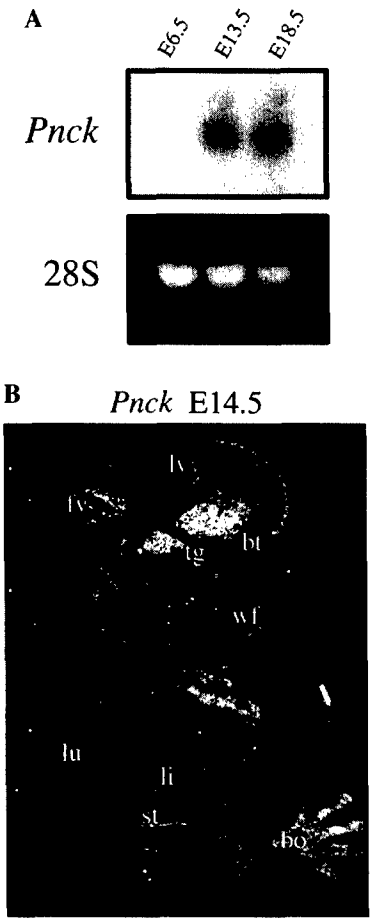

FIG. 5. Expression of *Pnck* during murine embryogenesis. (**A**) Northern hybridization analysis of 3 μg of poly(A)$^+$ RNA isolated from day E6.5, E13.5, and E18.5 embryos hybridized with a ^{32}P-labeled DNA probe specific for the 3′-UTR of *Pnck*. (**B**) *In situ* hybridization analysis of *Pnck* mRNA expression in the murine embryo. Sections of embryos at day 14.5 of gestation were hybridized with an ^{35}S-labeled *Pnck* antisense RNA probe. No signal over background was detected in serial sections hybridized with a sense *Pnck* probe. bo, bone; bt, basal telencephalon; fv, fourth ventricle; li, liver; lu, lung; lv, lateral ventricle; st, stomach; tg, trigeminal ganglion; wf, whisker hair follicle. Magnification: 10×. Exposure time was 6 weeks.

heterogeneous with highly expressing cells found adjacent to nonexpressing cells (Figs. 6J and 6K). *Pnck* is expressed throughout the ovary, but is preferentially localized in the thecal cell layers immediately surrounding the corpora lutea (Figs. 6H and 6I). In the testis, *Pnck* is expressed at high levels in mature spermatids residing at the center of seminiferous tubules and, to a lesser extent, in cells located adjacent to the basement membrane (Figs. 6D and 6E). Finally, in the dorsolateral prostate, *Pnck* mRNA is detected in a stromal layer of cells immediately surrounding the prostatic epithelial ducts. As in other tissues, *Pnck* expression in this compartment is spatially heterogeneous (Figs. 6L and 6M).

DISCUSSION

We have described the cloning, chromosomal localization, and developmental expression pattern of *Pnck*,

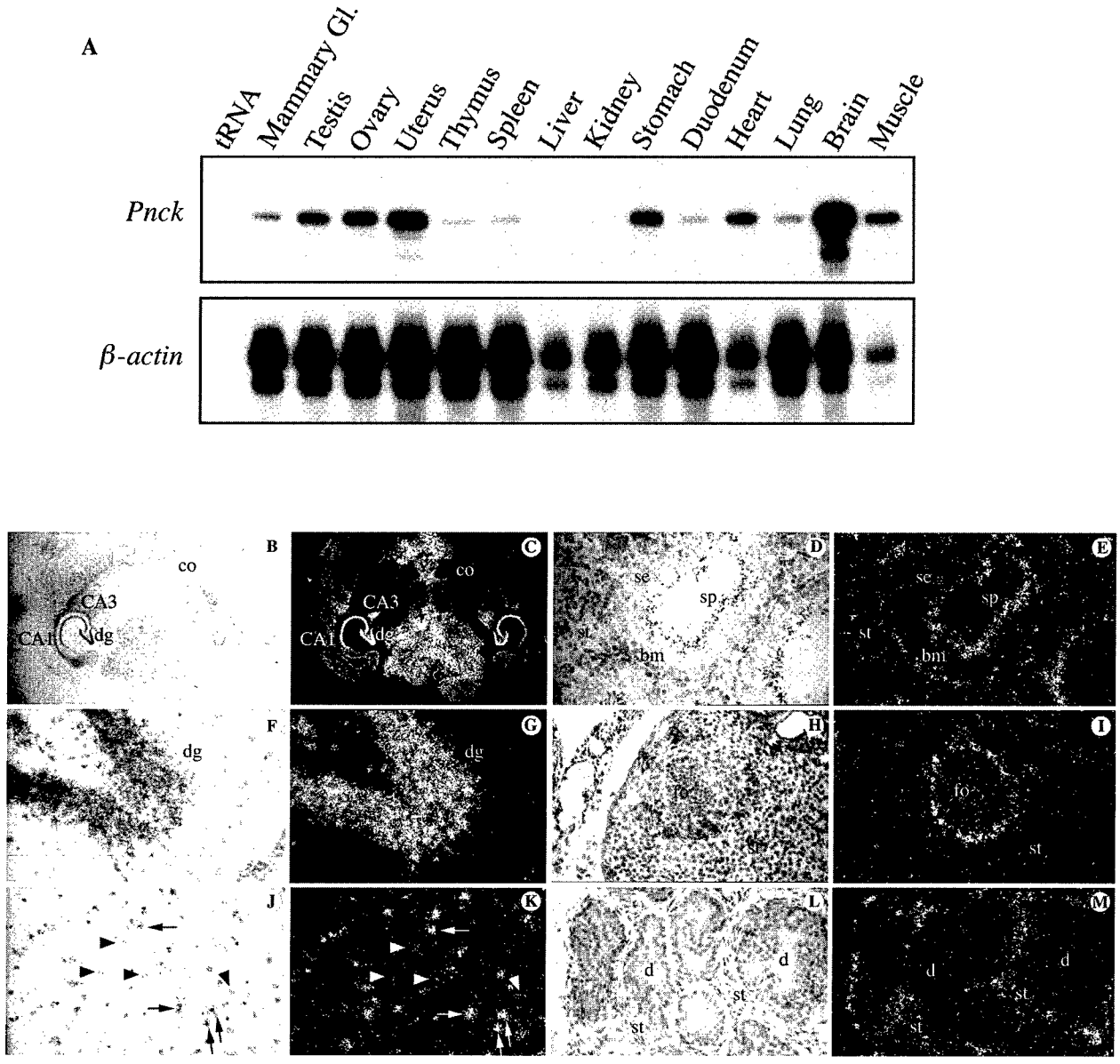

FIG. 6. Expression of *Pnck* in adult tissues. (**A**) RNase protection analysis of *Pnck* mRNA expression in tissues of the adult mouse. RNase protection analysis was performed using 30 μg of RNA isolated from the indicated murine tissues using antisense RNA probes specific for *Pnck* as well as for *β-actin* as an internal control. tRNA was used as a negative control for nonspecific hybridization. (**B–M**) Spatial localization of *Pnck* expression in tissues of the adult mouse. Bright-field (**B, D, F, H, J, L**) and dark-field (**C, E, G, I, K, M**) photomicrographs of *in situ* hybridization analysis performed on sections of brain (**B, C, F, G, J, K**), testis (**D, E**), ovary (**H, I**), and prostate (**L, M**), hybridized with an ^{35}S-labeled *Pnck* antisense probe. No signal over background was detected in serial sections hybridized with a sense *Pnck* probe. Arrows and arrowheads indicate *Pnck* expressing and *Pnck* nonexpressing cells, respectively. bm, basement membrane; CA1 and CA3, regions of the hippocampus; co, cortex; d, duct; dg, dentate gyrus; fo, follicle; se, seminiferous tubule; sp, spermatids; st, stroma. Magnifications: 10× (**B, C**), 300× (**D–M**). Exposure times were 6–7 weeks.

a new member of the CaM kinase family of serine/threonine kinases that is most closely related to CaMKI. *Pnck* expression during embryogenesis is developmentally regulated and tissue-specific with highest levels of expression detected in the developing brain, bone, and gut. Interestingly, *Pnck* expression in adult animals is both tissue-specific and markedly heterogeneous. *Pnck* expression is restricted to specific compartments within several tissues. Moreover, within these compartments, *Pnck* expression is heterogeneous with expressing cells found adjacent to non-expressing cells. The *Pnck* locus maps to the mouse X chromosome in a region of conserved synteny with human chromosome Xq28, a region associated with several mental retardation syndromes. In aggregate, our data suggest that *Pnck* may be involved in a variety of developmental processes.

Within the catalytic and regulatory domains conserved among all CaM kinases, Pnck is most closely related to the multifunctional CaM kinases, CaMKI, CaMKIV, and members of the CaMKII subfamily. CaMKI is a monomeric kinase that is expressed in

multiple tissues and is reported to phosphorylate several substrates including synapsin, the cystic fibrosis transmembrane conductance regulator, and transcription factors such as the cyclic AMP response element-binding protein, CREB and ATF-1 (Lukas *et al.,* 1998; Nairn and Piciotto, 1994; Nastluk and Nairn, 1996; Sheng *et al.,* 1991). CaMKIV is located in the nucleus and has been proposed to mediate CaM-induced changes in gene expression (Jensen *et al.,* 1991a; Sun *et al.,* 1996). In contrast to CaMKI and IV, which function as monomers, CaMKII forms 300- to 600-kDa multimers composed of different combinations of α, β, γ, and δ subunits (Schulman, 1993). While the α and β subunits are expressed predominantly in brain, the γ and δ CaMKII subunits are expressed ubiquitously (Hanley *et al.,* 1987; Lin *et al.,* 1987; Tobimatsu and Fujisawa, 1989; Tobimatsu *et al.,* 1988).

Functional analysis of CaM kinase mutants as well as crystal structure information has been used to define amino acids involved in the regulation of this family of molecules (Goldberg *et al.,* 1996; Haribabu *et al.,* 1995; Yokokura *et al.,* 1995). Carboxyl-terminal to their catalytic domain, CaM kinases possess a regulatory region that is composed of an autoinhibitory domain and a CaM-binding domain. Current evidence suggests that CaM binding disrupts an interaction between the autoinhibitory domain and the kinase catalytic domain, thereby resulting in kinase activation. In contrast to other CaM kinases, the activities of CaMKI and CaMKIV are dependent upon phosphorylation by a CaM-dependent kinase kinase, CaMKK (Haribabu *et al.,* 1995; Tokumitsu *et al.,* 1994, 1995). Since Pnck may be regulated in a manner similar to that of other CaM kinases, particularly CaMKI, structural homologies between Pnck and other CaM kinases may help elucidate the mechanisms by which Pnck activity is regulated.

The homology between Pnck and CaMKI raises the issue of whether Pnck should be classified as a CaMKI family member. Currently, the only widely recognized CaM kinase subfamily is that of CaMKII. Primary amino acid sequences of CaMKII subfamily members are typically greater than 90% identical in the catalytic and regulatory domains and actually function together in a multiprotein complex. In contrast, while the 70% amino acid identity in the catalytic domain between Pnck and CaMKI is greater than that between Pnck and other CaM kinases, the similarity between Pnck and CaMKI is significantly less than the approximately 90% identity observed between CaMKII family members. Moreover, there is currently no evidence to suggest that CaMKI family members function as subunits in a manner analogous to CaMKII subfamily members. As such, while the primary amino acid sequence of Pnck is most similar to that of CaMKI, it is unclear at present whether this kinase should be classified as a CaMKI family member.

While this work was in progress, the rat homologue of *Pnck* was described and shown to be expressed as two isoforms, tentatively named *CaMKIβ1* and *CaMKIβ2* (Naito *et al.,* 1997). Similar to the clones isolated for *Pnck, CaMKIβ1* and *CaMKIβ2* differ in their 5′-UTR regions and are homologous to *Pnck* clones V1 and U7, respectively. However, unlike the full-length clones isolated for *Pnck,* CaMKIβ1 contains a unique carboxyl-terminal coding region that appears to result from an alternative splicing event. Whether this form exists in the mouse remains to be determined. Northern hybridization analysis using a probe encompassing portions of the highly conserved kinase domain and regulatory region of *CaMKIβ* isoforms detected a 1.8-kb band exclusively in brain, whereas a 4.0-kb band was detected in all other tissues. RT-PCR analysis detected approximately equal levels of *CaMKIβ1* in all tissues examined in the rat, including tissues that we find to express low or undetectable levels of *Pnck* in the mouse as determined by RNase protection analysis using a probe specific for the 3′-UTR of *Pnck.* Insofar as the tissue-specific expression pattern of *Pnck* has been confirmed by *in situ* hybridization analysis, it is possible that cross-hybridization of the *CaMKIβ* probe used for Northern hybridization with other CaM kinases or the nonlinear nature of RT-PCR may underlie the discrepancy between the tissue-specific pattern of *Pnck* expression and the ubiquitous expression pattern reported for its rat homologue, *CaMKIβ.*

Several CaM kinases are expressed at high levels in the brain including CaMKI, CaMKII, and CaMKIV (Lukas *et al.,* 1998; Miyano *et al.,* 1992; Picciotto *et al.,* 1995). In fact, it has been estimated that CaMKII accounts for approximately 2% of all protein found in the hippocampus (Hanson and Schulman, 1992). It is therefore perhaps not surprising that *Pnck* is also expressed at high levels in the murine brain both in the adult and during embryogenesis. In the adult brain, *Pnck* is expressed at highest levels in the hippocampus and dentate gyrus, two areas of the brain involved in learning and memory.

While expression of CaMKI, CaMKIV, and isoforms of CaMKII has been reported in tissues other than the brain, a physiological role for these enzymes in other tissues has not been described. Similar to these multifunctional kinases, *Pnck* is expressed in a variety of tissues other than the brain. Moreover, in most tissues examined, *Pnck* is expressed in a spatially heterogeneous manner with expression restricted to a subset of cells. Although little is known about the role of CaM or CaM kinases in development, evidence suggests that both CaM and CaM kinases may play such a role. Point mutations in the *Drosophila* calmodulin gene result in defects in development with phenotypes ranging from pupal lethality to ectopic wing vein formation and melanotic scales on the cuticle (Nelson *et al.,* 1997). Additionally, CaMKIV has been implicated in T cell development based upon its regulation in the thymus during T cell development (Krebs *et al.,* 1997). The observation that *Pnck* expression is developmentally regulated and spatially restricted to distinct compartments of the

ovary, testis, prostate, and brain suggests that *Pnck* may play a biological role in these tissues. As such, the elucidation of signaling pathways in which *Pnck* is involved may shed light on the broader physiological role played by CaM kinases.

We have mapped the murine gene encoding *Pnck* to within 2.2 cM of *Il1rak* in the central region of the X chromosome. The observation that *Il1rak,* as well as markers that lie within 2.2 cM on either side of *Il1rak,* have been mapped to human chromosome Xq28 strongly suggests that the human homologue of *Pnck* will map to Xq28 as well. Chromosome Xq28 is one of the most densely mapped regions of the human chromosome, and several distinct mental retardation syndromes including Fragile X and X-linked mental retardation (XLMR) have been mapped to this region (Knight *et al.,* 1993; Lubs *et al.,* 1999). Interestingly, the only biological role described for any of the multifunctional CaM kinases is that of CaMKII in learning and memory. This suggests that CaM kinases may play an important role in signal transduction pathways controlling cognitive function (Silva *et al.,* 1992a, b; Soderling, 1993). In addition to mental retardation, many of these syndromes include phenotypes such as short stature, cleft palate, altered hand or digit size, and sterility (Lubs *et al.,* 1999). Given that *Pnck* is expressed at high levels in the brain, developing bone, ovary, and testis, it will be interesting to determine whether *Pnck* plays a role in one or more of these Xq28-linked syndromes.

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Developmental role of the SNF1-related kinase Hunk in pregnancy-induced changes in the mammary gland

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SUMMARY

The steroid hormones 17β-estradiol and progesterone play a central role in the pathogenesis of breast cancer and regulate key phases of mammary gland development. This suggests that developmental regulatory molecules whose activity is influenced by ovarian hormones may also contribute to mammary carcinogenesis. In a screen designed to identify protein kinases expressed in the mammary gland, we previously identified a novel SNF1-related serine/threonine kinase, Hunk (hormonally upregulated Neu-associated kinase). During postnatal mammary development, *Hunk* mRNA expression is restricted to a subset of mammary epithelial cells and is temporally regulated with highest levels of expression occurring during early pregnancy. In addition, treatment of mice with 17β-estradiol and progesterone results in the rapid and synergistic upregulation of *Hunk* expression in a subset of mammary epithelial cells, suggesting that the expression of this kinase may be regulated by ovarian hormones. Consistent with the tightly regulated pattern of *Hunk* expression during pregnancy, mammary glands from transgenic mice engineered to misexpress *Hunk* in the mammary epithelium manifest temporally distinct defects in epithelial proliferation and differentiation during pregnancy, and fail to undergo normal lobuloalveolar development. Together, these observations suggest that Hunk may contribute to changes in the mammary gland that occur during pregnancy in response to ovarian hormones.

Key words: Mammary gland, Protein kinase, Transgenic, Cell differentiation, Hormone, Mouse, Hunk

INTRODUCTION

A wealth of epidemiological evidence indicates that ovarian hormones play a crucial role in the etiology of breast cancer (Kelsey et al., 1993). Specifically, the observations that early menarche, late menopause and postmenopausal hormone replacement therapy are each associated with increased breast cancer risk, whereas early oophorectomy is associated with decreased breast cancer risk, have led to the hypothesis that breast cancer risk is proportional to cumulative estradiol and progesterone exposure (Henderson et al., 1988; Pike et al., 1993). As such, elucidating the mechanisms by which hormones contribute to mammary carcinogenesis is a central goal of breast cancer research.

In addition to their roles in the pathogenesis of breast cancer, estradiol and progesterone are the principal steroid hormones responsible for regulating the development of the mammary gland during puberty, pregnancy and lactation (Topper and Freeman, 1980). For example, estradiol action is required for epithelial proliferation and ductal morphogenesis during puberty, whereas progesterone action is required for ductal arborization and alveolar differentiation during pregnancy (Bocchinfuso and Korach, 1997; Humphreys et al., 1997; Topper and Freeman, 1980). The effects of estradiol and progesterone in a given tissue are ultimately determined by the activation and repression of their respective target genes. Consequently, understanding the effects of estradiol and progesterone in the breast will require the identification of downstream targets of these hormones, and the analysis of these targets will undoubtedly contribute to our understanding of both mammary development and carcinogenesis.

Protein kinases function as molecular switches in signal transduction pathways that regulate cellular processes such as proliferation and differentiation. Accordingly, aberrant expression or mutations in several members of the protein kinase family have been shown to be involved in the pathogenesis of breast cancer both in humans and in rodent model systems (Cardiff and Muller, 1993; Cooper, 1990; Di Fiore et al., 1987; Muller et al., 1988). Moreover, overexpression of protein kinases such as ERBB2/neu in

human breast cancers has been shown to provide prognostic information relevant to clinical outcome and response to therapy (Klijn et al., 1993; Slamon et al., 1987, 1989). Given the important roles played by protein kinases in development and carcinogenesis, we previously carried out a PCR-based screen to identify kinase family members expressed either during mammary development or in epithelial cell lines derived from different transgenic mouse models of breast cancer (Chodosh et al., 1999, 2000). A total of 41 protein kinases were identified in this screen including 33 tyrosine kinases and eight serine/threonine kinases, three of which were novel (Chodosh et al., 2000; Gardner et al., 2000a,b; Stairs et al., 1998).

One of these novel kinases, Hunk, is an 80 kDa putative serine/threonine kinase that bears homology to the SNF1 family of protein kinases (Gardner et al., 2000b). Several members of this family, including SNF1 in *Saccharomyces cerevisiae* and AMP-activated protein kinase in mammals, regulate metabolic changes that occur in response to nutritional and environmental stresses (Hardie et al., 1994). Other SNF1-related kinases, including PAR1, MARK1, MARK2 and MARK3/KP78/C-TAK1, have been implicated in the regulation of developmental processes (Bohm et al., 1997; Guo and Kemphues, 1995; Peng et al., 1997; Ruiz et al., 1994). For example, in both *Caenorhabditis elegans* and *Drosophila, par-1* is required for the establishment of anterior-posterior axis formation during embryogenesis (Guo and Kemphues, 1995; Shulman et al., 2000). Analogously, a mammalian homolog of PAR1, MARK2/EMK, is asymmetrically localized in polarized epithelial cells, and expression of a dominant negative form of MARK2 disrupts cell polarity (Bohm et al., 1997). Consistent with a developmental role for this molecule, disruption of murine *Emk/MARK* results in dwarfism, pituitary defects and hypofertility (Bessone et al., 1999). Additional SNF1-related kinases such as *Msk* and SNRK have been implicated in vertebrate development on the basis of their temporal and spatial patterns of expression (Becker et al., 1996; Ruiz et al., 1994). These data suggest a role for SNF1 family members in development in higher eukaryotes.

We have previously described the cloning, activity and chromosomal localization of *Hunk* and have shown that *Hunk* mRNA expression is temporally and spatially regulated during embryonic development (Gardner et al., 2000b). In the developing embryo, *Hunk* is expressed at high levels within a subset of organs during mid-gestation and is downregulated in some, but not all, tissues prior to parturition. As in the embryo, *Hunk* expression in the adult mouse is tissue specific, with highest levels observed in ovary, lung and brain. Interestingly, within multiple tissues *Hunk* mRNA expression is restricted to specific compartments and within these compartments is further restricted to a subset of cells. For example, *Hunk* expression in the duodenum is limited to a subset of epithelial cells in duodenal crypts, whereas little or no expression is observed in more differentiated cells of the duodenal epithelium. The tissue-specific, temporally regulated and spatially restricted pattern of *Hunk* expression in the mouse suggest a developmental role for this kinase in multiple tissues.

In this report, we investigate the role of *Hunk* in the mammary gland. During postnatal mammary development, *Hunk* mRNA expression is spatially restricted to a subset of epithelial cells and is tightly regulated, with highest levels of expression occurring early in pregnancy. Moreover, treatment of mice with 17β-estradiol and progesterone results in the rapid and synergistic induction of *Hunk* expression in the mammary epithelium, suggesting that *Hunk* upregulation during early pregnancy may be due to increases in circulating levels of ovarian hormones. Finally, misexpression of *Hunk* in the mammary epithelium of MMTV-*Hunk* transgenic mice results in decreased proliferation and impaired differentiation of alveolar epithelial cells during distinct periods of pregnancy and lactation. Taken together, our data suggest that Hunk may contribute to mammary development by regulating pregnancy-induced changes in the alveolar epithelium that occur in response to estrogen and progesterone.

MATERIALS AND METHODS

Animal and tissue preparation

FVB mice were housed under barrier conditions with a 12-hour light/dark cycle. Mammary glands from pregnant females were harvested at specified timepoints after timed matings. Female mice were housed with male mice every third night and day 0.5 was defined as noon of the day on which a vaginal plug was observed. Gestational stage was confirmed by analysis of embryos. Transgenic mothers were housed with wild-type mothers immediately after parturition to ensure pup survival and equivalent suckling stimuli. Both transgenic and wild-type females were observed to nurse pups. For experiments involving chronic hormone treatment, adult female FVB mice were subject to bilateral oophorectomy and allowed to recover for two weeks prior to hormonal injections that were administered as previously described (Marquis et al., 1995). For short-term hormone administration experiments, four-month-old virgin female FVB mice were injected subcutaneously with either phosphate buffered saline (PBS) or a combination of 5 mg progesterone in 5% gum arabic and 20 μg of 17β-estradiol in PBS. Four animals from each treatment group were sacrificed 24±1 hours after injection. Tissues used for RNA analysis were snap frozen on dry ice. Tissues used for in situ hybridization analysis were embedded in OCT compound.

For whole mount analysis, number four mammary glands were spread on glass slides and fixed for 24 hours in 10% neutral buffered formalin. Glands were subsequently immersed in 70% ethanol for 15 minutes followed by 15 minutes in deionized water prior to staining in 0.05% Carmine/0.12% aluminum potassium sulfate for 24-48 hours. Glands were dehydrated sequentially in 70%, 90% and 100% ethanol for 10 minutes each, and then cleared in toluene or methyl salicylate overnight. For histological analysis, mammary glands were fixed as above and transferred to 70% ethanol prior to paraffin embedding. Sections 5 μm thick were cut and stained with Hematoxylin and Eosin. For BrdU analysis, animals were injected with 50 μg BrdU per g total bodyweight two hours before sacrifice followed by fixation and paraffin embedding as above.

Generation of MMTV-*Hunk* transgenic mice

A full-length cDNA clone, G3, encoding *Hunk*, was digested with *Sma*I and *Spe*I to liberate a 3.2 kb fragment containing the complete coding sequence for *Hunk* (GenBank Accession number AF167987). This fragment was cloned downstream of the mouse mammary tumor virus long terminal repeat (MMTV LTR) into the multiple cloning site of pBS-MMTV-pA (E. Gunther, unpublished), which consists of the MMTV LTR upstream of the H-ras leader sequence (Huang et al., 1981) and SV40 splicing and polyadenylation signals. Linearized plasmid DNA was injected into fertilized oocytes harvested from superovulated FVB mice. Tail-derived DNA was prepared as described (Hogan et al., 1994). Mice were genotyped by Southern hybridization analysis and by two independent PCR reactions designed to amplify a region within the SV40 portion of the transgene,

and a region spanning the junction between *Hunk* and SV40 sequences. A portion of the *Gapdh* (glycelaldehyde-3-phosphate dehydrogenase) locus was amplified as a positive control for PCR reactions. Oligonucleotide primer sequences were Gapdh.F, CTCACTCAAGATTGTCAGCAATGC; Gapdh.B, AGGGTTTCT-TACTCCTTGGAGGC; SV40.F, CCTTAAACGCCTGGTGCTA-CGC; SV40.B, GCAGTAGCCTCATCATCACTAGATGG; Hunk.F, CTTTCTTTTTCCCCTGACC; PolyA.B, ACGGTGAGTAGCGTC-ACG. Southern hybridization analysis of tail-derived genomic DNA digested with *Spe*I was performed according to standard methods using a probe specific to the SV40 portion of the transgene. Four founder mice were identified harboring the MMTV-*Hunk* transgene in tail-derived DNA that passed the transgene to offspring in a Mendelian fashion. These were screened for transgene expression by Northern hybridization and RNase protection analysis. One founder line, MHK3, was identified that expressed the MMTV-*Hunk* transgene at high levels. Of note, a subset of transgene-positive MHK3 animals was found not to express the MMTV-*Hunk* transgene. All MHK3 non-expressing animals were analyzed by Southern hybridization analysis to confirm transgene presence and the expected MHK3-specific integration site.

RNA preparation and analysis

RNA preparation, northern hybridization and labeling of cDNA probes was performed as previously described (Marquis et al., 1995). The ^{32}P-labeled cDNA probe for *Hunk* encompassed nucleotides 275 to 793 (GenBank Accession number AF167987). Probes for milk protein gene expression were: β-casein, nt 181-719 (GenBank Accession number X04490); κ-casein, nt 125-661 (GenBank Accession number M10114); lactoferrin, nt 993-2065 (GenBank Accession number D88510); WAP, nt 131-483 (GenBank Accession number X01158) and ε-casein, nt 83-637 (GenBank Accession number V00740).

Ribonuclease protection analysis was performed as described using body-labeled antisense riboprobes specific to nucleotides 276-500 of *Hunk* and 1142-1241 of β-actin (GenBank Accession number X03672; Marquis et al., 1995). In order to distinguish transgenic from endogenous *Hunk* expression in MHK3 animals, RNase protection analysis was performed using an antisense riboprobe spanning the 3′ end of the *Hunk* cDNA and the 5′ end of the SV40 polyadenylation signal sequence. A β-actin antisense riboprobe was added to each reaction as an internal control. Signal intensities were quantitated by phosphorimager analysis (Molecular Dynamics).

In situ hybridization was performed as described (Marquis et al., 1995; Rajan et al., 1997) using a PCR template containing nucleotides 276 to 793 of *Hunk*. Exposure times were 6 weeks in all cases.

Protein analysis

Generation of anti-Hunk antisera, immunoblotting and immunoprecipitation were performed as described (Gardner et al., 2000b). Protein was extracted from mammary glands by dounce homogenization in EBC buffer as described (Gardner et al., 2000b). For immunoprecipitation, 500 μg of protein (3 mg/ml) was precleared with 1/10 vol of 1:1 protein A-sepharose in PBS overnight at 4°C. Precleared lysates were incubated overnight at 4°C in EBC (50 mM Tris-HCl, pH 7.9; 120 mM NaCl; 0.5% NP40) plus 5% Tween 20 (Biorad) with or without affinity-purified antisera raised against the C terminus of Hunk (0.4 μg/ml). Immune complexes were precipitated by incubating with 40 μl of 1:1 protein A-sepharose in PBS for 1 hour at 4°C. Complexes were washed sequentially with EBC plus 5% Tween 20, EBC (2×), and PBS (2×). One-fifth of the precipitated complexes were used in an in vitro kinase reaction as previously described with 5 μM ATP and 0.5 μg/μl histone H1 (Gardner et al., 2000b). The remaining precipitate was electrophoresed on a 10% SDS-PAGE gel, transferred onto a PVDF membrane, and immunoblotted with an antibody against the C terminus of Hunk as described (Gardner et al., 2000b).

Immunohistochemistry

Mammary glands from nulliparous wild-type and MHK3 transgenic females were fixed in 4% paraformaldehyde overnight and transferred to 70% ethanol prior to paraffin embedding. 5 μm sections were dewaxed in xylene and sequentially rehydrated in 100%, 95% and 70% ethanol, followed by PBS. Sections were incubated in Antigen Unmasking Solution (Vector) for 30 minutes at 100°C and then transferred to PBS at room temperature (RT). Sections were incubated for 2 hours at RT with antibody raised against the C terminus of Hunk, washed in PBS (×3), then incubated with 1:500 biotinylated goat anti-rabbit antibody (Vector) in 1%BSA/PBS for 30 minutes at RT. After washing in PBS (×3), slides were incubated in a 1:250 dilution of Avidin (Vector) for 15 minutes at RT and washed in PBS (3×). NBT and BCIP substrate addition was performed in alkaline phosphate buffer for 3 minutes according to manufacturer instructions (BMB). Sections were counterstained for 10 minutes in 0.5% (w/v) Methyl Green in 1.0 M NaOAc, pH 4.0.

BrdU

Paraffin-embedded 5 μm sections were dewaxed as above, pretreated in 2N HCl for 20 minutes at RT, washed in 0.1 M borate buffer, pH 8.5 (×2) and rinsed in PBS. BrdU immunohistochemistry was performed using the Vectastain Elite ABC Kit (Vector Laboratories), rat anti-BrdU IgG (Vector), and a secondary biotinylated rabbit anti-rat IgG antibody according to manufacturer instructions. Sections were counterstained with Methyl Green as described above. The areas of BrdU-positive and -negative nuclei were quantitated by color segmentation analysis of digitally captured images using Image-Pro Plus software (Media Cybernetics). The percentage of BrdU-positive epithelial cells was determined after normalizing nuclear area to the average nuclear size of either BrdU positive or negative cells.

RESULTS

Hunk, initially termed *Bstk1*, was identified as a 207 bp RT-PCR product isolated from an epithelial cell line derived from a mammary adenocarcinoma arising in an MMTV-*neu* transgenic mouse (Chodosh et al., 1999, 2000; Gardner et al., 2000b). *Bstk1* was subsequently renamed *Hunk* to reflect the upregulation of this kinase in the mammary gland both during pregnancy and in response to ovarian hormones, as well as the preferential expression of this kinase in transgenic murine mammary epithelial cell lines overexpressing the *neu/Erbb2* oncogene (this report and data not shown).

Hunk expression is developmentally regulated in the mammary gland

RNase protection analysis was used to determine the temporal pattern of *Hunk* expression during the postnatal development of the murine mammary gland (Fig. 1A). Mammary glands were harvested from male FVB mice, virgin mice at developmental time points prior to puberty (2 weeks), during puberty (5 weeks) and after puberty (10 weeks and 15 weeks), as well as from mice during early, mid and late pregnancy (day 7, 14 and 20), lactation (day 9), and postlactational regression (days 2, 7 and 28). This analysis revealed that steady-state levels of *Hunk* mRNA were low and remained relatively constant throughout virgin development. During early pregnancy (day 7), when alveolar buds begin to proliferate rapidly and differentiate, *Hunk* mRNA levels underwent a dramatic increase and then returned to baseline by mid-pregnancy (Fig. 1A,B). The apparent decline in β-actin expression seen by RNase protection analysis during late

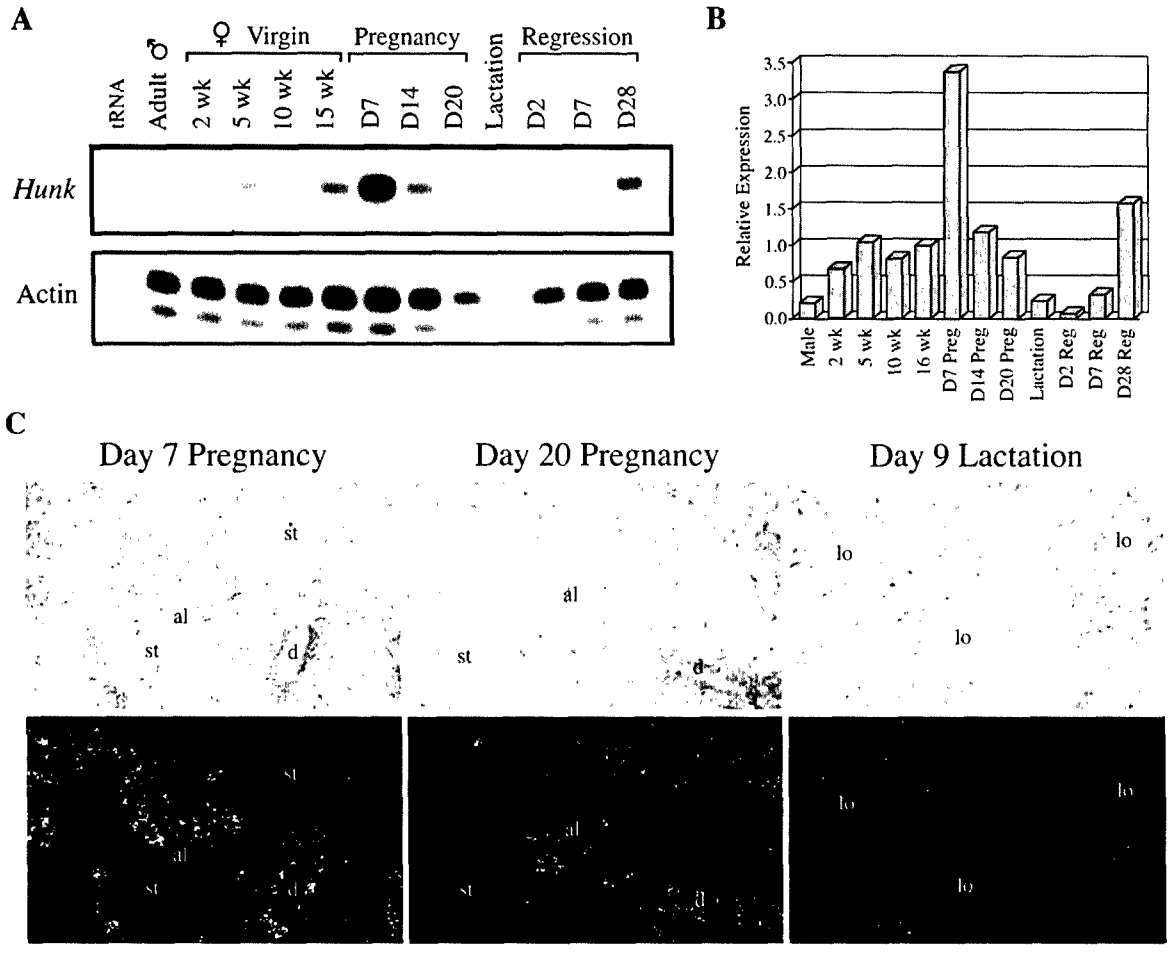

Fig. 1. Temporal regulation of *Hunk* expression during mammary gland development. (A) RNase protection analysis of *Hunk* mRNA expression during postnatal developmental of the murine mammary gland. 40 μg of total RNA isolated from mammary glands at the indicated timepoints was hybridized to a ^{32}P-labeled antisense RNA probe specific for *Hunk*. A ^{32}P-labeled antisense RNA probe specific for β-actin was included in the same hybridization reaction as an internal loading control. (B) Phosphorimager analysis of RNase protection analysis in A. *Hunk* expression was quantitated and normalized to β-actin expression to correct for dilutional effects due to large-scale increases in expression of milk protein genes during late pregnancy and lactation. Expression levels are shown relative to adult virgin (15 wk). (C) In situ hybridization analysis of *Hunk* expression during pregnancy and lactation. Bright-field (top panel) and dark-field (bottom panel) photomicrographs of mammary gland sections from day 7 pregnant, day 20 pregnant or day 9 lactating animals hybridized with an ^{35}S-labeled *Hunk*-specific antisense probe. No signal over background was detected in serial sections hybridized with a sense *Hunk* probe. Exposure times were identical for all dark-field photomicrographs to illustrate changes in *Hunk* expression during pregnancy. al, alveoli; d, duct; lo, lobule; st, adipose stroma.

pregnancy, lactation and early postlactational regression results from a dilutional effect that is due to large-scale expression of genes for milk proteins during these developmental stages (Buhler et al., 1993; Gavin and McMahon, 1992; Marquis et al., 1995). Normalization of *Hunk* expression to β-actin to control for this dilutional effect confirmed that *Hunk* expression returned to baseline levels by mid-pregnancy and decreased further during lactation and early postlactational regression (Fig. 1B). An essentially identical expression profile was observed during pregnancy when *Hunk* mRNA levels were normalized to cytokeratin 18, an epithelial-specific marker, indicating that developmental changes in *Hunk* expression are not the result of changes in epithelial cell content in the gland during pregnancy (data not shown). This conclusion is supported by the finding that *Hunk* mRNA expression levels decreased from day 7 to day 14 of pregnancy, despite ongoing increases in epithelial cell content that occur during this stage of development. Furthermore, changes in *Hunk* expression did not appear to be the result of increased cellular proliferation, since the pattern of *Hunk* expression observed during pregnancy did not correlate with levels of epithelial proliferation that, unlike *Hunk* expression, remained elevated during mid-pregnancy (see Fig. 5B).

In order to determine whether the observed pregnancy-induced changes in *Hunk* mRNA expression levels represent global changes in expression throughout the mammary gland, or changes in expressing subpopulations of cells, in situ hybridization was performed (Fig. 1C and data not shown). Consistent with the results from RNase protection analysis, in situ hybridization confirmed that *Hunk* expression in the mammary gland was highest at day 7 of pregnancy and decreased progressively throughout the remainder of pregnancy and lactation. This analysis also revealed that *Hunk* was expressed exclusively in the epithelium throughout mammary gland development and that *Hunk* upregulation during pregnancy appeared to result from both the upregulation

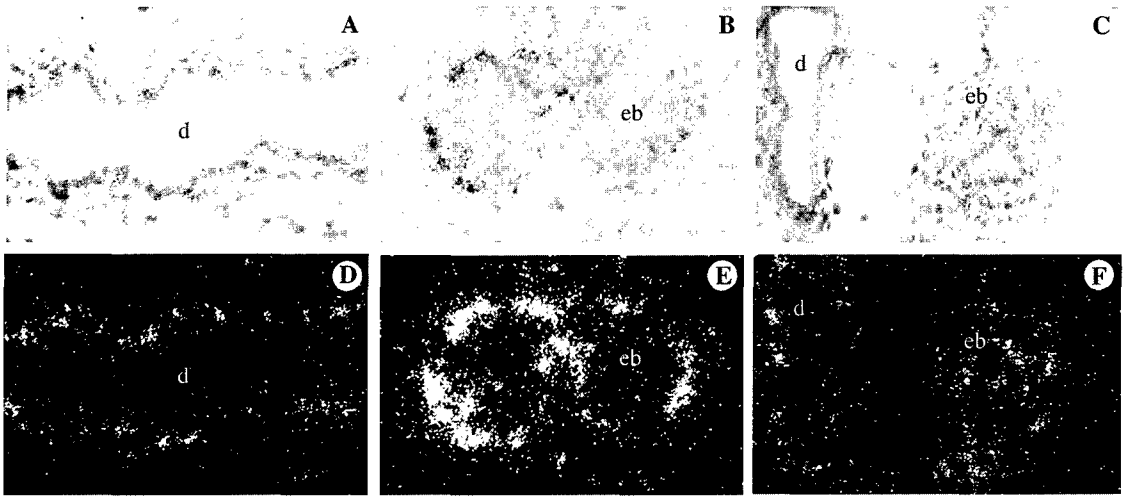

Fig. 2. Heterogeneous expression of *Hunk* in the mammary epithelium (A-F). In situ hybridization analysis of *Hunk* expression in the virgin mammary gland using an ^{35}S-labeled *Hunk*-specific antisense probe. Bright-field (A-C) and dark-field (D-F) photomicrographs of in situ hybridization analysis performed on mammary gland sections from 5-week-old nulliparous females. In all cases note the heterogeneous expression pattern of *Hunk* in both epithelial ducts (A,C,D,F) and terminal end buds (B,C,E,F). No signal over background was detected in serial sections hybridized with a sense *Hunk* probe. Exposure times were optimized for each dark-field panel. d, duct; eb, terminal end bud.

of *Hunk* in a subset of cells and an increase in the proportion of *Hunk*-expressing epithelial cells (Fig. 1C and data not shown).

The observation that cells highly expressing *Hunk* are found adjacent to non-expressing cells indicates that, as in other organs of the adult mouse, *Hunk* expression in the mammary gland is spatially restricted (Figs 1C and 2). This heterogeneous expression pattern is particularly striking in terminal end buds and epithelial ducts of the adolescent gland (Fig. 2). These data suggest that the murine mammary epithelium is composed of *Hunk*-expressing and *Hunk* non-expressing cell types.

Hunk expression is regulated by ovarian hormones

The observation that *Hunk* mRNA levels in the mammary gland increase during pregnancy suggests that the expression of this gene may be modulated by estrogen and progesterone. In order to test this possibility, oophorectomized FVB mice were treated for fourteen days with 17β-estradiol alone, progesterone alone, or a combination of both hormones. Intact (sham) and oophorectomized, non-hormone treated (OVX) animals were used for comparison.

Hunk mRNA levels were quantitated by RNase protection analysis of RNA prepared from mammary glands or uteri pooled from at least 10 animals in each experimental group (Fig. 3). Steady-state *Hunk* mRNA levels were approximately fourfold lower in the mammary glands of oophorectomized mice compared with intact mice, suggesting that maintenance of basal levels of *Hunk* expression in the mammary glands of nulliparous mice requires ovarian hormones (Fig. 3A). Treatment of oophorectomized animals with 17β-estradiol alone increased *Hunk* mRNA expression but to levels below those observed in intact animals, whereas treatment with progesterone alone increased *Hunk* mRNA expression to levels comparable with those observed in intact animals. In contrast, treatment of oophorectomized animals with both 17β-estradiol and progesterone resulted in a 14-fold increase in the level of *Hunk* mRNA relative to control oophorectomized animals and a 3-fold increase relative to intact animals, similar to increases in *Hunk* expression observed during early pregnancy. These observations suggest that the increase in *Hunk* mRNA expression observed in the mammary gland during early pregnancy may result, either directly or indirectly, from increases in circulating levels of estrogens and progesterone.

Treatment of mice with ovarian hormones also affected *Hunk* expression in the uterus (Fig. 3B). Steady-state *Hunk* mRNA levels were nearly two-fold higher in oophorectomized animals compared with intact mice suggesting that circulating levels of 17β-estradiol may repress *Hunk* expression in the uteri of nulliparous mice. Consistent with this suggestion, treatment of oophorectomized animals with 17β-estradiol either alone or in combination with progesterone decreased *Hunk* expression to levels below those observed in either intact or oophorectomized animals. In contrast to findings in the mammary gland, progesterone treatment had little if any effect on *Hunk* expression in the uterus. These results suggest that the increase in *Hunk* mRNA expression observed in the uterus following oophorectomy is due, either directly or indirectly, to loss of tonic inhibition of *Hunk* expression by estradiol. The observation that the combination of estradiol and progesterone has opposing effects on *Hunk* expression in the mammary gland and uterus is consistent with the opposing physiological effects of these hormones on proliferation and differentiation in these tissues.

The effects of estradiol and progesterone on *Hunk* expression in the mammary gland and uterus were confirmed by in situ hybridization analysis performed on tissues from the experimental animals described above (Fig. 3D and data not shown). Consistent with RNase protection results, oophorectomy resulted in a marked decrease in *Hunk* mRNA expression in the mammary epithelium and the combination of 17β-estradiol and progesterone resulted in a synergistic increase in *Hunk* expression. Reminiscent of *Hunk* expression during early pregnancy, the upregulation of *Hunk* mRNA levels

in oophorectomized animals treated with a combination of 17β-estradiol and progesterone occurred in a subset of epithelial cells in both ducts and developing alveolar buds.

Since the above experiments involved the chronic administration of hormones, sufficient time elapsed during hormone treatment for significant developmental changes to occur in both the mammary glands and uteri of oophorectomized animals. As such, these experiments do not distinguish whether changes in *Hunk* expression reflect direct regulation by ovarian hormones, or are a consequence of the changes in epithelial proliferation and differentiation that occur in response to the chronic administration of ovarian hormones. To address this issue, intact mice were treated with a combination of 17β-estradiol and progesterone for 24 hours before sacrifice (Fig. 3C). Mice treated in such a manner do not develop the marked morphological changes characteristic of long-term hormone administration. Analysis of *Hunk* mRNA expression levels in these mice revealed a pattern similar to that observed in mice treated chronically with hormones. Within 24 hours of the administration of 17β-estradiol and progesterone, steady-state levels of *Hunk* mRNA increased in the mammary gland and decreased in the uterus. These findings suggest that the regulation of *Hunk* expression by estradiol and progesterone is not solely a consequence of changes in mammary and uterine tissue architecture that occur in response to chronic hormone treatment, but rather may result from direct regulation by these hormones.

Generation of MMTV-*Hunk* transgenic animals

The tightly regulated expression of *Hunk* observed in the mammary gland during pregnancy and in response to ovarian hormones suggests the possibility that *Hunk* may play a role in mediating pregnancy-induced changes in the mammary gland. To test this hypothesis, transgenic mice overexpressing *Hunk* in a mammary-specific fashion were generated using the MMTV LTR. Activity of the MMTV LTR is upregulated in mammary epithelial cells during pregnancy and lactation in response to rising levels of prolactin, progesterone and glucocorticoids. Since endogenous *Hunk* expression is heterogeneous and is transiently upregulated during early pregnancy, MMTV-driven expression of *Hunk* in transgenic mice would be predicted to alter the temporal and spatial profile of *Hunk* expression in the mammary gland.

A cDNA encoding the full-length Hunk protein was cloned downstream of the MMTV LTR and injected into superovulated FVB mice. One of four founder lines, MHK3, was found to express the *Hunk* transgene at high levels in the mammary gland and was therefore studied further (Fig. 4A). The tissue specificity of transgene expression in the MHK3 line was determined by RNase protection analysis using a transgene-specific probe (Fig. 4B). This analysis confirmed that nulliparous MHK3 transgenic females express high levels of the MMTV-*Hunk* transgene in the mammary gland and lower but detectable levels of transgene expression in the spleen, salivary gland, lung and thymus, as has been observed for other MMTV transgenic mouse models.

The hormonally responsive nature of the MMTV LTR often results in low levels of expression in the mammary glands of nulliparous transgenic animals and high levels of transgene expression during pregnancy that peak during lactation. In contrast, MHK3 animals express high levels of

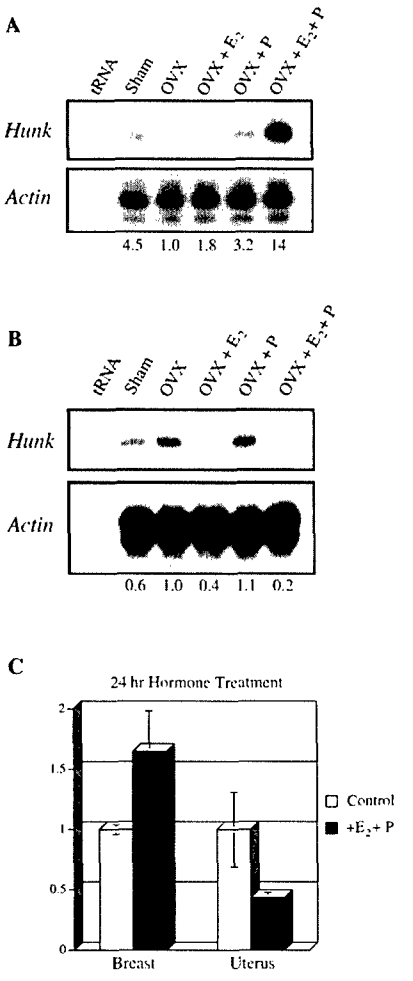

Fig. 3. Ovarian hormones alter *Hunk* expression in vivo. *Hunk* mRNA expression in mammary glands and uteri of mice treated with ovarian hormones. (A,B) Tissues were harvested from either intact females (sham) or oophorectomized females that received daily subcutaneous injections of either vehicle alone (OVX), 17β-estradiol (OVX+E_2), progesterone (OVX+P), or both 17β-estradiol and progesterone (OVX+E_2+P) for fourteen days. Each sample represents a pool of at least 10 mice. 20 μg of total RNA isolated from the mammary glands (A) or uteri (B) of treated animals was hybridized overnight with ^{32}P-labeled antisense RNA probes specific for *Hunk* and β-actin. Signal intensities were quantitated by phosphorimager analysis and *Hunk* expression was normalized to β-actin expression levels. *Hunk* expression relative to expression in oophorectomized (OVX) controls is shown below each lane. (C) Quantitation of *Hunk* expression in mammary glands and uteri from intact FVB female mice killed 24 hours after injection with PBS (control; light shaded boxes) or a combination of 5 mg progesterone in 5% gum arabic and 20 μg of 17β-estradiol in PBS (+E_2+P; dark shaded boxes). RNase protection analysis was performed on either 20 μg (breast) or 40 μg (uterus) of total RNA using ^{32}P-labeled antisense RNA probes specific for *Hunk* and β-actin. *Hunk* expression was quantitated by phosphorimager analysis and normalized to β-actin. Values are shown relative to control animals. Each bar represents the average of four animals±s.e.m. for each group. (D) In situ hybridization analysis of *Hunk* expression in mammary gland sections from oophorectomized mice treated with hormones as described in A. Dark-field exposure times were identical in all cases. al, alveoli; d, duct; st, adipose stroma.

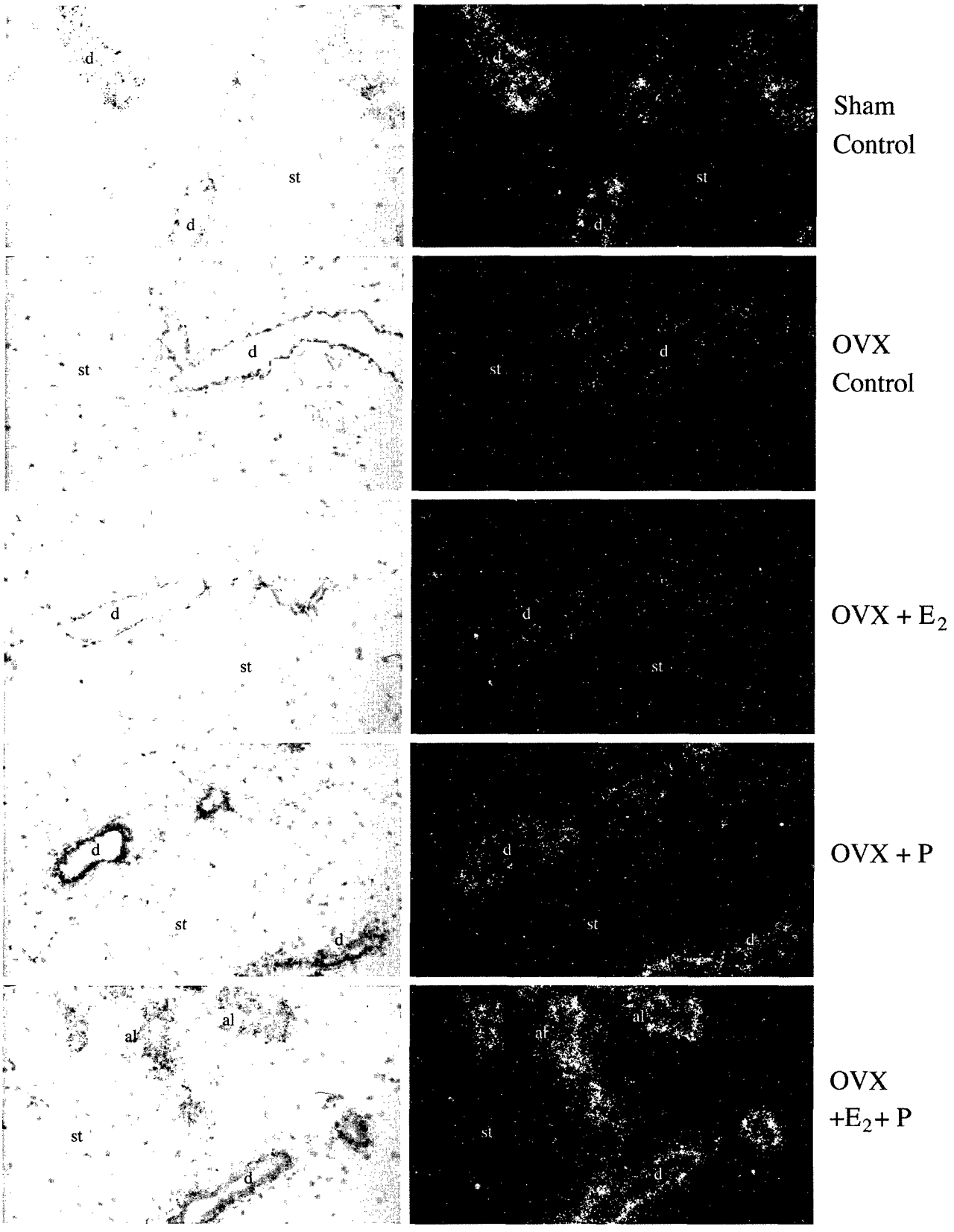
d
st
d
Sham
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Fig. 3D

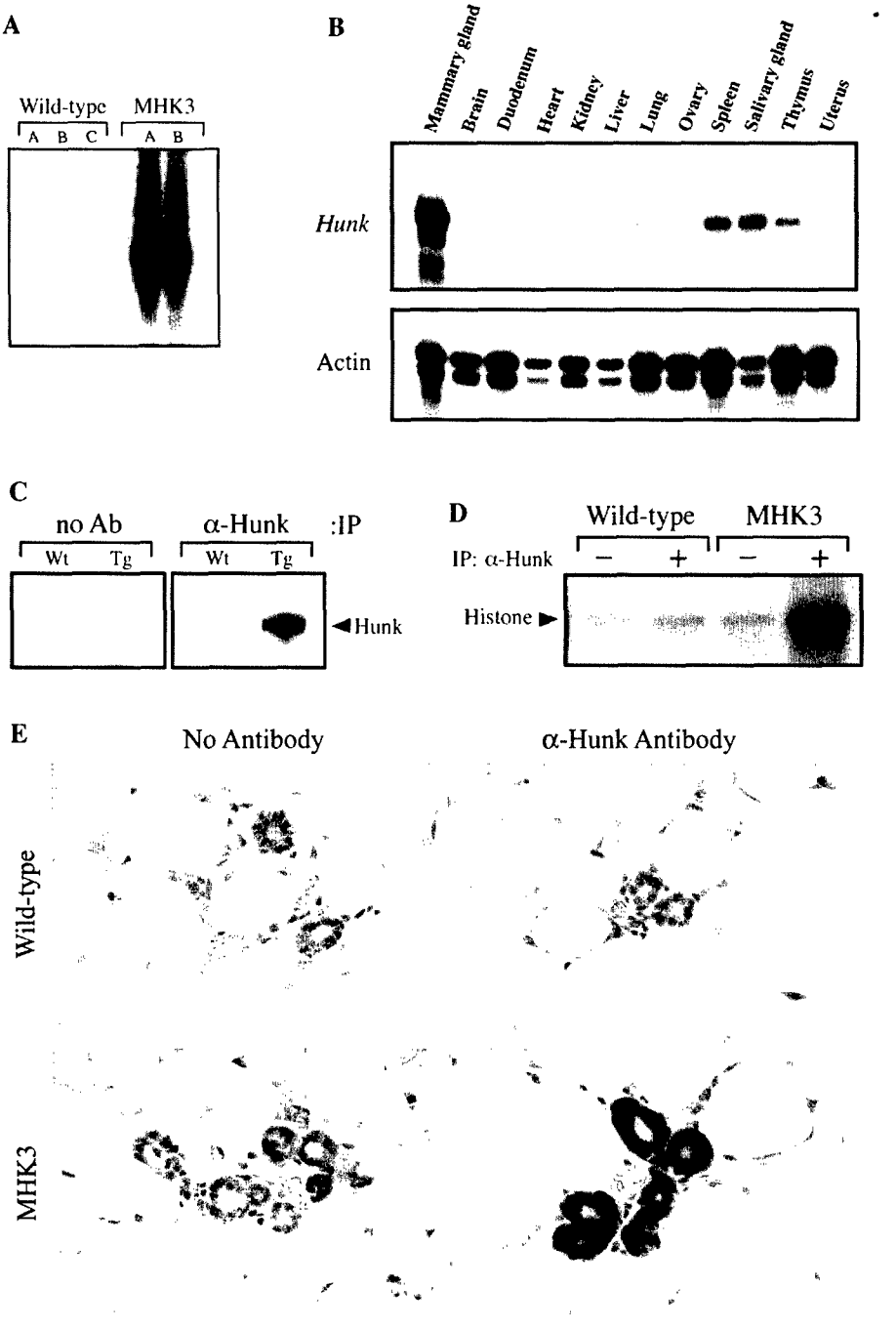


Fig. 4. MMTV-*Hunk* transgene expression in MHK3 transgenic mice. The MMTV LTR was used to generate transgenic mice misexpressing *Hunk* in a mammary-specific manner. (A) Northern hybridization analysis of MMTV-*Hunk* transgene expression in mammary glands from 7- to 9-week-old nulliparous wild-type or MHK3 transgenic mice using a ^{32}P-labeled probe specific for *Hunk*. The detected mRNA transcript corresponds to the expected size of the MMTV-*Hunk* transgene. (B) RNase protection analysis of MMTV-*Hunk* transgene expression in organs from a 7-week-old nulliparous MHK3 transgenic female mouse. A ^{32}P-labeled antisense RNA probe spanning the junction of the 3′ end of the *Hunk* cDNA and the 5′ end of the SV40 polyadenylation signal was used to specifically detect transgene expression in 20 μg of total RNA. A ^{32}P-labeled antisense RNA probe for β-actin was used in the same reaction to control for RNA loading and sample processing. (C) Immunoprecipitation of Hunk protein from lactating MHK3 transgenic animals. Affinity-purified antisera raised against the C terminus of Hunk (α-Hunk) was incubated with 500 μg of protein extract prepared from mammary glands harvested from either MHK3 transgenic (Tg) or wild-type (Wt) mice during lactation. A control reaction was performed without antisera (no Ab). Immunoprecipitated protein was analyzed by immunoblotting using C terminal anti-Hunk antisera. The expected migration of Hunk is indicated. (D) In vitro kinase assay of anti-Hunk immunoprecipitates. Histone H1 was used as an in vitro kinase substrate for protein immunoprecipitated with (+) or without (−) anti-Hunk antisera from extracts containing equal amounts of protein as in (C). The relative migration of histone H1 is indicated. (E) Immunohistochemical analysis of Hunk protein expression in MHK3 transgenic mice. Anti-Hunk antisera from (C) and (D) above was used to detect Hunk protein in sections from paraffin-embedded mammary glands harvested from 14-week-old nulliparous wild-type or MHK3 transgenic females. A control assay was performed by omitting primary antisera from the protocol. Detection reaction times were identical in all cases.

the MMTV-*Hunk* transgene in the nulliparous state. In addition, MMTV-*Hunk* transgene expression levels in mammary glands from pregnant or lactating MHK3 animals were found to vary less than threefold relative to nulliparous MHK3 animals, a range of expression that is far less than that typically found in MMTV-based transgenic mouse models (data not shown). Together, these data indicate that MMTV-*Hunk* transgene expression is high relative to endogenous *Hunk* expression during all stages of postnatal mammary development.

To determine if *Hunk* mRNA levels in transgenic mice resulted in changes in Hunk protein levels, antisera specific to Hunk were used to analyze Hunk expression levels in extracts prepared from lactating mammary glands of MHK3 transgenic and wild-type mice (Fig. 4C; Gardner et al., 2000b). Western analysis of immunoprecipitated Hunk using Hunk-specific antisera revealed increased amounts of Hunk protein in extracts prepared from transgenic when compared with wild-type mammary glands (Fig. 4C). The inability to detect Hunk protein in extracts from wild-type lactating glands was consistent with the barely detectable levels of endogenous *Hunk* mRNA expression during this developmental stage (Fig.

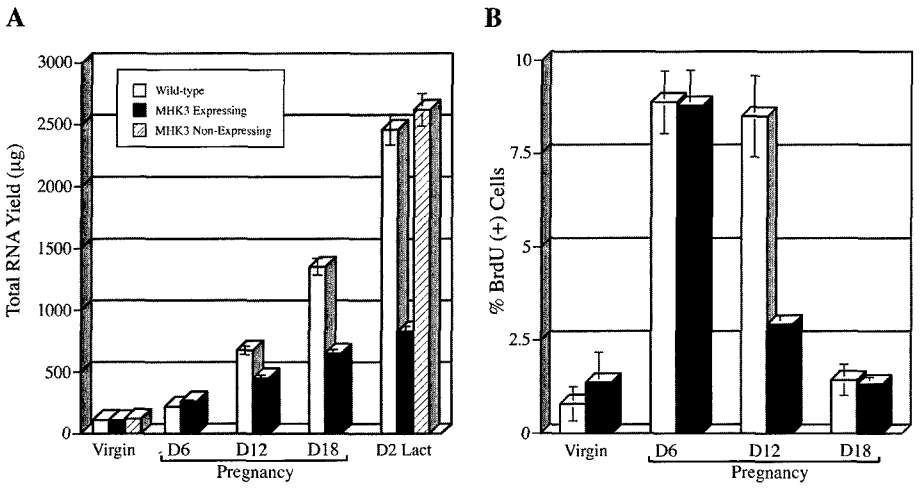


Fig. 5. Effect of Hunk overexpression on RNA content and mammary epithelial proliferation. (A) Amount of total RNA isolated from either wild-type (light-shaded boxes), expressing MHK3 transgenic (dark-shaded boxes), or non-expressing MHK3 transgenic (hatched boxes) female mice during mammary development. Total RNA was isolated from number 3 and number 5 mammary glands harvested from female mice at the indicated developmental timepoints. The average total RNA yield for each group is represented as the mean±s.e.m. At least three mice were analyzed from each group. A significant difference in RNA content was observed between wild-type and transgenic mammary glands at day 18.5 of pregnancy and day 2 of lactation (*t*-test, P=0.047 and 0.0007, respectively). (B) Relative percentage of BrdU-positive epithelial cells in the mammary glands of wild-type and MHK3 transgenic mice during development. Two hours before being killed, mice received injections of 50 µg BrdU per g total body weight. Following fixation and paraffin embedding, BrdU incorporation was detected using an anti-BrdU antibody followed by ABC detection method (Vector). The fraction of BrdU-positive and negative epithelial cells was determined by quantitative analysis using Phase 3 Imaging Software. At least four different fields per animal and three animals per timepoint were analyzed for BrdU incorporation. A significant difference in the fraction of BrdU-positive cells was observed between wild-type and transgenic mammary glands only at day 12.5 of pregnancy (*t*-test, P=0.004).

1). Conversely, MMTV-*Hunk* transgene expression was very high during lactation (data not shown).

In order to demonstrate that Hunk-associated kinase activity is also elevated in MHK3 transgenic animals, in vitro kinase assays were performed. Hunk was immunoprecipitated from protein extracts prepared from the lactating mammary glands of wild-type or transgenic mice as above (Fig. 4D). Control immunoprecipitation reactions were carried out in the absence of anti-Hunk antisera. The resulting immunoprecipitates were incubated with γ-^{32}P-ATP and histone H1. As predicted, based on the relative quantities of Hunk in these extracts, Hunk-associated kinase activity was substantially greater in immunoprecipitates prepared from transgenic when compared with wild-type mammary glands. These experiments confirm that MHK3 transgenic animals manifest increased levels of both Hunk protein and Hunk-associated kinase activity.

To investigate the spatial pattern of Hunk protein expression in MHK3 transgenic animals, immunohistochemistry was performed on mammary glands harvested from nulliparous transgenic and wild-type female mice (Fig. 4E). Consistent with high levels of MMTV-Hunk mRNA expression in MHK3 mice, this analysis revealed high levels of Hunk protein expression in transgenic compared with wild-type mammary glands. As described for other MMTV transgenic models, exogenously expressed Hunk was restricted to the epithelium of MHK3 mice. In addition, Hunk expression in the mammary epithelium of MHK3 animals was found to be relatively homogeneous, unlike the heterogeneous patterns of transgene expression observed in other MMTV transgenic models or the heterogeneous expression of endogenous *Hunk* mRNA. These data indicate that compared with wild-type animals, MHK3 transgenic animals overexpress Hunk in a mammary epithelial-specific and relatively homogenous manner.

Notably, some MHK3 transgenic animals did not express the MMTV-*Hunk* transgene. The presence of the MHK3-specific transgene integration site was confirmed by Southern hybridization analysis for all non-expressing MHK3 transgenic mice. A similar type of transgene silencing has been observed in other MMTV transgenic models (Betzl et al., 1996; Sternlicht et al., 1999).

Hunk overexpression results in impaired lactation

Consonant with the hypothesis that Hunk plays a role in mammary gland development during pregnancy, we initially noted that the number of pups successfully reared by MHK3 transgenic females was significantly reduced compared with wild-type animals, with many pups dying within 1-2 days of birth independent of pup genotype. In contrast, offspring of transgenic males mated to wild-type females displayed survival rates comparable with those observed for offspring of wild-type crosses. These observations suggested that the inability to successfully rear pups was due to a defect in the ability of MHK3 transgenic females to lactate.

Consistent with the presence of a lactation defect in MHK3 mice, we initially noted that mammary glands from pregnant or lactating transgenic animals contained lower amounts of RNA compared with their wild-type counterparts. The amount of total RNA isolated from wild-type murine mammary glands is highly dependent upon developmental stage and can increase almost two orders of magnitude from the nulliparous state to the peak of lactation. The dramatic increase in RNA content during pregnancy and lactation is due to a combination of increased epithelial cell number and increased milk protein gene expression by individual alveolar epithelial cells. To confirm our initial observations regarding reduced RNA content in MHK3 mammary glands, we determined the yield of total RNA isolated from mammary glands harvested from either wild-type or MHK3 transgenic females during mammary development (Fig. 5A). As expected, in wild-type animals this analysis revealed an approximately 20-fold increase in RNA yield from lactating when compared with nulliparous mammary glands. In contrast, the increase in RNA yield over this developmental interval was significantly lower in MHK3 transgenic glands, with the difference between wild-

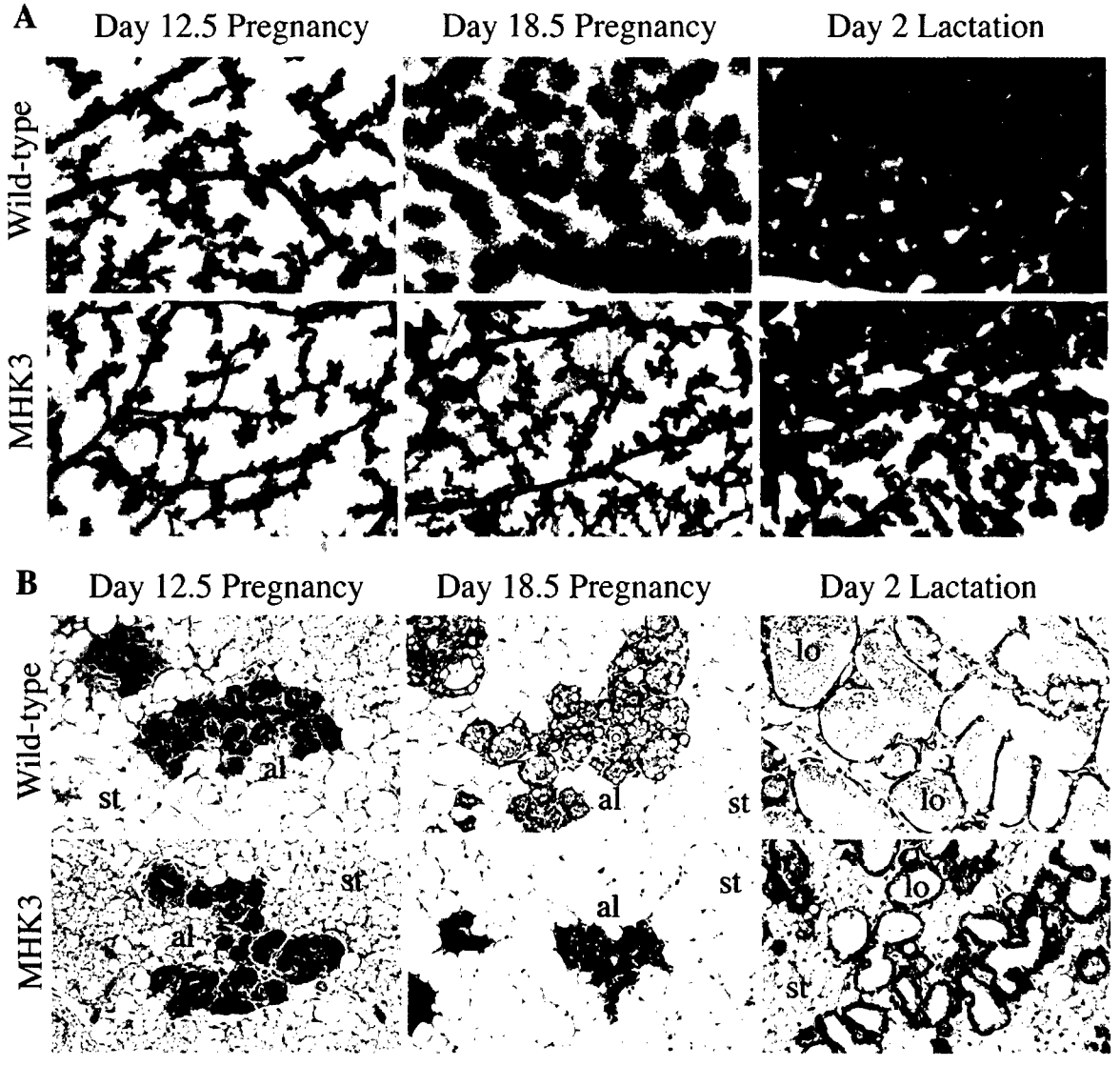


Fig. 6. Morphological defects in MHK3 transgenic mice during late pregnancy and lactation. Number four mammary glands from MHK3 transgenic and wild-type females were harvested at day 12.5 and day 18.5 of pregnancy, and day 2 of lactation. At least three transgene-expressing mice and three wild-type mice were analyzed for each timepoint. A representative photomicrograph is shown for each group. (A) Whole-mount analysis of transgenic and wild-type mammary glands at the indicated timepoints. Harvested glands were fixed and stained with Carmine dye in order to visualize epithelial ducts and alveoli. (B) Representative Hematoxylin and Eosin-stained sections of paraffin-embedded transgenic and wild-type mammary glands. al, alveoli; lo, lobule; st, adipose stroma.

type and transgenic glands becoming more pronounced towards late-pregnancy and lactation (wild-type versus transgenic, *t*-test P=0.047 (day 18.5 of pregnancy) and P=0.0007 (day 2 of lactation). In fact, at day 2 of lactation only one third of the total amount of RNA was isolated from transgenic when compared with wild-type glands. Non-expressing MHK3 transgenic females exhibited RNA yields indistinguishable from wild-type animals, indicating that the reduction in RNA observed in MHK3 animals was dependent upon expression of the *Hunk* transgene (Fig. 5A). These data suggest the possibility of impaired mammary development in MHK3 animals during pregnancy and lactation.

Hunk overexpression decreases epithelial proliferation during mid-pregnancy

Lobuloalveolar development during pregnancy involves both proliferation and differentiation of alveolar epithelial cells. Alveolar cell proliferation occurs primarily during the first two trimesters of pregnancy, while alveolar differentiation occurs in a graded and progressive manner throughout pregnancy. To determine whether the decrease in RNA yield obtained from MHK3 transgenic glands during pregnancy is related to a decrease in cellular proliferation in these mice, we compared BrdU incorporation rates in epithelial cells from wild-type and transgenic mammary glands (Fig. 5B). Wild-type and MHK3 transgenic female mice at different developmental stages were pulse labeled with BrdU before sacrifice and the percentage of BrdU-positive epithelial cells was determined by quantitative analysis of anti-BrdU-stained sections. As predicted based upon the similar morphology of wild-type and transgenic mammary glands in nulliparous animals (data not shown), no significant difference in the percentage of BrdU-positive cells

was observed between wild-type and transgene-expressing mammary glands harvested from nulliparous animals. Moreover, a dramatic increase in epithelial proliferation was observed at day 6.5 of pregnancy both in wild-type and transgenic animals relative to nulliparous females. In contrast, at day 12.5 of pregnancy, epithelial proliferation rates remained high in wild-type glands but dropped markedly in glands from MHK3 animals (wild-type versus transgenic at day 12.5, *t*-test, P=0.004). By comparison, no differences in epithelial proliferation rates were observed between wild-type and transgenic glands at day 18.5 of pregnancy. Furthermore, no differences in apoptosis rates were observed between wild-type and MHK3 transgenic glands during virgin development, pregnancy or lactation, as evidenced by similar levels of TUNEL-positive cells (data not shown). Since MMTV-*Hunk* transgene expression levels in MHK3 animals are roughly comparable in the mammary gland throughout pregnancy and do not coincide with the observed defect in proliferation, we conclude that *Hunk* overexpression inhibits mammary epithelial proliferation specifically during mid-pregnancy.

Hunk overexpression impairs lobuloalveolar development

Our finding of increased pup death among offspring of MHK3 females, together with the decreased RNA content of mammary glands from lactating MHK3 animals, suggested that MHK3 female glands may have a defect in lobuloalveolar development. To address this hypothesis directly, MHK3 transgenic females were sacrificed at different stages of pregnancy and lactation for morphological analysis. Analysis of both whole mounts and Hematoxylin and Eosin stained sections at day 6.5 and day 12.5 of pregnancy revealed no obvious morphological differences between the mammary glands of wild-type and MHK3 transgenic animals, despite the fact that epithelial cell proliferation is markedly impaired in MHK3 female mice at day 12.5 of pregnancy (Figs 5B and 6, and data not shown). In contrast, marked morphological differences were observed between wild-type and transgenic animals at day 18.5 of pregnancy. Analysis of whole mounts and Hematoxylin and Eosin stained sections at this stage of development consistently showed decreased lobuloalveolar development in MHK3 transgenic animals (Fig. 6). In addition to their larger size, alveoli in wild-type mice at day 18.5 of pregnancy contained copious amounts of lipid, whereas those of MHK3 mice did not.

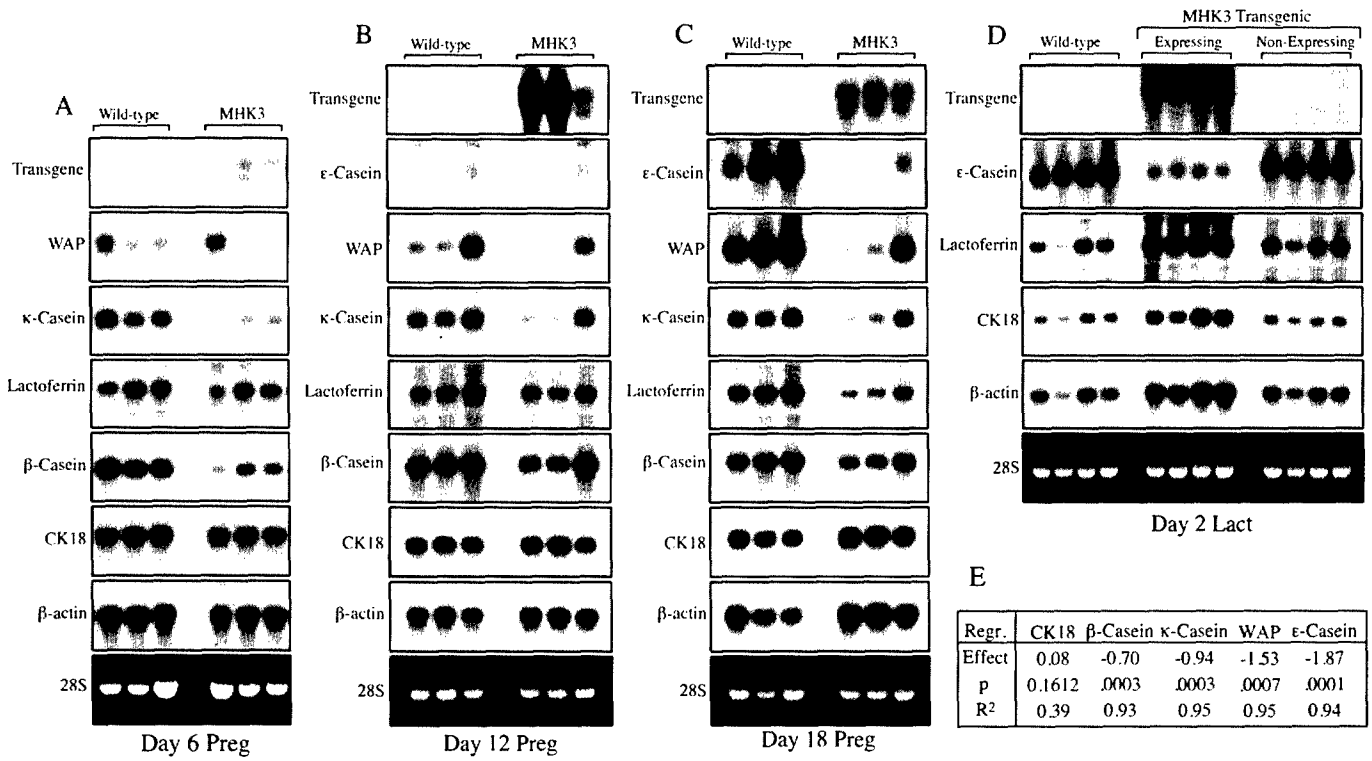
In addition to the abnormalities observed at day 18.5 of pregnancy, decreased lobuloalveolar development was also observed in MHK3 females at day 2 of lactation. Normally during lactation the mammary gland is filled with casein-secreting lobules such that by whole-mount analysis the gland is entirely opaque, and by histological analysis no white adipose tissue is seen (Fig. 6). In contrast, lobuloalveolar units in lactating Hunk-overexpressing transgenic animals were smaller and appeared less developed by whole-mount analysis compared with wild-type and non-expressing MHK3 females (Fig. 6A and data not shown). Consequently, only half of the mammary fat pad of lactating MHK3 mice was occupied by secretory alveoli (Fig. 6B). While this may be due in part to decreased epithelial cell proliferation observed during mid-pregnancy, morphometric analysis of Hematoxylin and Eosin stained sections from MHK3 mice at day 18.5 of pregnancy and day 2 of lactation revealed that compared with their wild-type counterparts, the mammary glands of MHK3 animals consist of a normal number of alveoli that are uniformly smaller and less differentiated morphologically, rather than a smaller number of morphologically normal alveoli. (Fig. 6B and data not shown). Moreover, alveoli in lactating transgenic animals were less distended with milk when compared with wild-type glands. In contrast, similar analyses performed on the mammary glands of non-expressing MHK3 transgenic animals during lactation revealed no morphological defects (data not shown). These observations suggest that dysregulated expression of *Hunk* impairs terminal differentiation of the mammary gland during late pregnancy and lactation in a manner potentially distinct from the observed defect in epithelial proliferation.

Hunk overexpression inhibits mammary epithelial differentiation

The dramatic changes in epithelial differentiation that occur in the mammary gland during lobuloalveolar development are reflected on a molecular level by the tightly regulated and temporally ordered expression of genes for milk proteins (Robinson et al., 1995). While steady-state mRNA levels for each of these genes typically increase throughout pregnancy, each gene undergoes a maximal increase in expression at a characteristic time during pregnancy. These differential expression profiles permit individual genes to be classified as early (β-casein), intermediate (κ-casein, lactoferrin), late-intermediate (WAP) or late (ε-casein) markers of mammary epithelial differentiation (Robinson et al., 1995; C. D'Cruz, unpublished). As such, the expression of these genes can be used as a molecular correlate for the extent of mammary epithelial differentiation. Accordingly, analysis of temporal expression patterns of milk protein genes permits the degree of lobuloalveolar differentiation to be reproducibly and objectively determined at the molecular level.

To confirm that the defect in lobuloalveolar development observed in MHK3 transgenic mice included a defect in differentiation, and was not simply a consequence of reduced epithelial cell numbers, we examined the expression of a panel of molecular differentiation markers in wild-type and MHK3 animals during lobuloalveolar development. We reasoned that if the defect in lobuloalveolar development was solely due to reduced epithelial cell mass, then the absolute level of expression of milk protein genes in MHK3 animals should be similar to that observed in wild-type animals when normalized for epithelial content. Similarly, if alveolar cells present in MHK3 glands differentiate normally during pregnancy, then the levels of expression of early, mid and late differentiation markers relative to each other should be similar to that observed in wild-type animals. As such, the observation that the absolute levels of expression of multiple differentiation markers are reduced despite normalizing for epithelial content, or that the expression of these differentiation markers relative to each other is altered compared to wild-type animals, would indicate that mammary epithelial differentiation is impaired in MHK3 animals and is independent of the observed proliferation defect.

To determine whether MHK3 animals manifest a defect in differentiation in addition to the defect in proliferation demonstrated above, we determined mRNA expression levels



E

Regr.	CK18	β-Casein	κ-Casein	WAP	ε-Casein
Effect	0.08	-0.70	-0.94	-1.53	-1.87
p	0.1612	.0003	.0003	.0007	.0001
R^2	0.39	0.93	0.95	0.95	0.94

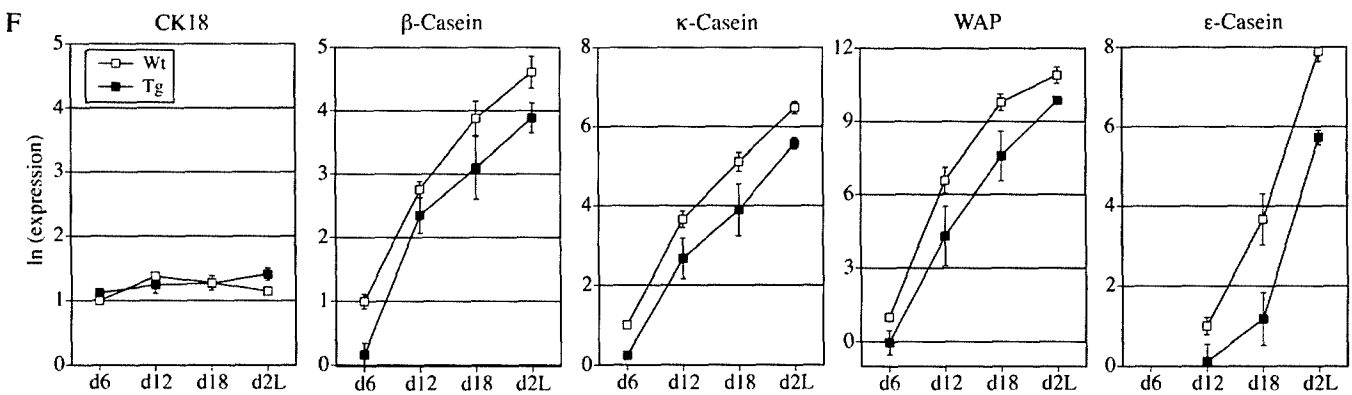


Fig. 7. Differentiation defects in MHK3 transgenic mice during pregnancy and lactation. (A-D) Northern analysis of gene expression for epithelial differentiation markers (β-casein, κ-casein, lactoferrin, WAP and ε-casein) in the mammary glands of wild-type or MHK3 transgene-expressing animals at day 6.5 of pregnancy (A), day 12.5 of pregnancy (B), day 18.5 of pregnancy (C) or at day 2 of lactation (D). Differentiation marker expression in the mammary glands of non-expressing MHK3 transgenic animals is also shown in D. β-actin expression is shown as a control for dilutional effects and the 28S ribosomal RNA band is shown as a loading control. (E) Multivariate regression analysis of (A-D) demonstrating the effects of transgene expression and developmental stage on the natural logarithm of cytokeratin 18 and expression levels of milk protein genes. All expression levels were normalized to β-actin. The average effect of transgene expression (Effect) on the expression of each milk protein gene is represented as the natural logarithm of the average fold-difference between transgenic and wild-type values. The respective *P* value (significance of transgene effect) is shown for each milk protein gene. Note that transgene expression has no effect on cytokeratin 18 expression and results in an average decrease in the expression levels of differentiation markers ranging from 2.0-fold (β-casein) to 6.5-fold (ε-casein). The R^2 value represents the degree to which the difference in the observed data from the null hypothesis is due to transgene expression. The *P* value for the significance of the regression model was < 0.01 for all differentiation markers shown.
(F) Phosphorimager quantitation of northern analyses in A-D. Expression levels of milk protein genes were normalized to β-actin expression and are shown on a logarithmic scale in arbitrary units relative to expression levels first detected in wild-type animals. Values are shown as the mean±s.e.m. for each point. The number of mice analyzed in each group is: 4 Wt, 5 Tg (d6.5); 3 Wt, 3 Tg (d12.5 and d18.5); and 4 Wt, 4 Tg, 4 non-expressing Tg (d2 Lact).

for a panel of early, intermediate and late markers of mammary epithelial differentiation in mammary glands from transgenic and wild-type animals during pregnancy and lactation (Fig. 7). Although few if any morphological differences were noted in transgenic mice before day 18.5 of pregnancy, when normalized to β-actin expression, steady-state levels of expression for all five milk protein genes were reduced in mammary glands from MHK3 transgenic mice compared with

wild-type mice beginning as early as day 6.5 of pregnancy and persisting throughout pregnancy and into lactation (Fig. 7). In contrast, expression levels of the epithelial cell marker, cytokeratin 18, did not differ significantly between wild-type and transgenic glands at any stage of pregnancy or lactation when normalized to β-actin expression (Fig. 7).

Accurate interpretation of gene expression levels in the mammary glands of mice bearing defects in lobuloalveolar development requires normalization to reference genes, such as β-actin, in order to control for differences in dilutional effects cause by expression of milk protein genes. Although β-actin levels do not change significantly on a per cell basis during pregnancy and lactation, the enormous contribution of the expression of milk protein genes to the total RNA pool results in an apparent decrease in the expression of reference genes when comparing equal amounts of total RNA (Figs 1 and 7). The magnitude of this dilutional effect correlates with the differentiation state of the mammary gland. Thus, the lower levels of expression of milk protein genes observed in the less differentiated MHK3 glands results in a less severe dilutional effect and apparent increases in β-actin and cytokeratin 18 expression in the mammary glands of MHK3 animals compared with wild-type animals at day 18 of pregnancy and day 2 of lactation. Therefore, in aggregate our findings indicate that the reduced expression of differentiation markers in MHK3 animals during pregnancy and lactation is not simply due to a reduction in epithelial cell content and suggests that mammary glands from *Hunk*-overexpressing transgenic mice are less differentiated than wild-type glands at each stage of lobuloalveolar development.

As further controls for these experiments, expression of milk protein genes was analyzed in non-expressing MHK3 transgenic females at day 2 of lactation (Figs 7 and 8, and data not shown). No differences in the expression either of cytokeratin 18 or of alveolar differentiation markers were observed between non-expressing MHK3 glands and glands from wild-type mice, consistent with the lack of morphological or functional defects in non-expressing MHK3 glands. Together, these findings strongly suggest that the abnormalities in mammary epithelial differentiation observed in MHK3 mice are due to MMTV-*Hunk* transgene expression rather than to site-specific integration effects such as the insertional disruption of an endogenous gene.

To analyze further the impact of MMTV-*Hunk* transgene expression on lobuloalveolar development, a multivariate regression analysis was performed on the above normalized gene expression data to quantitate the effects of transgene expression on mammary epithelial differentiation during a developmental interval from day 6.5 of pregnancy to day 2 of lactation (Fig. 7E,F). This analysis revealed that the expression of four epithelial differentiation markers (β-casein, κ-casein, WAP and ε-casein) was significantly lower in the mammary glands of transgenic animals compared with wild-type animals across all developmental time points. No differences were observed in cytokeratin 18 expression between wild-type and transgenic glands, confirming that normalization to β-actin expression was sufficient to control for differences in epithelial cell content. These results indicate that the mammary glands of MHK3 animals are significantly less differentiated than wild-type glands throughout pregnancy and into lactation.

Interestingly, the average reductions in mRNA expression

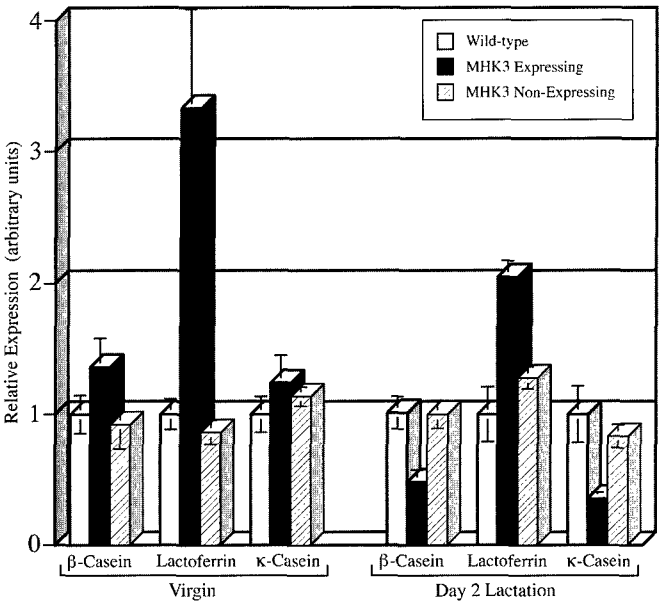


Fig. 8. Upregulation of lactoferrin expression at specific developmental stages in MHK3 mammary glands. Analysis of differentiation marker expression in mammary glands from either wild-type (light-shaded boxes), MHK3 transgene-expressing (dark-shaded boxes) or non-expressing MHK3 transgenic (hatched boxes) female mice during puberty or day 2 of lactation, as described in Fig. 7. Sample sizes were 16, 10 and 8, respectively for adolescent mice and 4 animals per group for lactation points. Northern hybridization analysis and quantitation was performed on 3 μg (virgin) or 5 μg (day 2 lactation) of total RNA isolated from mammary glands using ^{32}P-labeled cDNA probes specific for milk protein genes as indicated. Expression of these genes was normalized to that of β-actin. Wild-type expression values were set to 1.0 and are represented as the mean±s.e.m. for each group.

levels observed for the late differentiation marker, ε-casein (Tg effect=–1.87; 6.5-fold), and the late-intermediate differentiation marker, WAP (Tg effect=–1.53; 4.6-fold), were considerably more pronounced than the reductions in expression observed for the early differentiation marker, β-casein (Tg effect=–0.70; 2.0-fold), and the intermediate differentiation marker, κ-casein (Tg effect=–0.94; 2.6-fold) (Fig. 7). The observation that transgene expression had a greater effect on the expression of late differentiation markers compared with early differentiation markers suggests that late events in mammary epithelial differentiation are disproportionately affected during lobuloalveolar development in MHK3 mice. This finding is consistent with the morphological defects observed in these mice during late pregnancy.

Hunk upregulates lactoferrin expression in MHK3 mice

Surprisingly, while the expression of all 5 epithelial differentiation markers examined was reduced in the mammary glands of MHK3 transgenic animals throughout pregnancy, expression of the gene for lactoferrin was actually higher in transgenic animals compared with wild-type animals at day 2 of lactation (Figs 7D and 8). This finding prompted us to analyze the impact of Hunk overexpression on lactoferrin

expression in nulliparous MHK3 mice. Consistent with results obtained in lactating MHK3 animals, steady-state levels of lactoferrin mRNA were significantly higher in the mammary glands of nulliparous MHK3 expressing transgenic animals compared with either non-expressing MHK3 transgenic animals or age-matched nulliparous wild-type animals (Fig. 8). In contrast to lactoferrin, mRNA expression levels of the epithelial differentiation markers, β-casein, κ-casein, α-lactalbumin (Lalba – Mouse Genome Informatics), WDNM1 (Expi – Mouse Genome Informatics) and WAP, in adolescent nulliparous females were not significantly affected by *Hunk* overexpression (Fig. 8 and data not shown). Consistent with this finding, the rate of ductal elongation and extent of epithelial side-branching in mammary glands from 5- to 6-week-old nulliparous transgenic mice was comparable with that observed in wild-type mice as analyzed by whole-mount and histological analysis (data not shown). These observations suggest that Hunk does not cause precocious differentiation of the mammary gland during puberty, but may specifically activate pathways resulting in *lactoferrin* upregulation. Similarly, the observation that lactoferrin expression is upregulated in the mammary glands of lactating MHK3 animals, despite the global inhibitory effect of Hunk overexpression on mammary epithelial differentiation during late pregnancy and lactation, suggests that the effects of Hunk on lactoferrin expression are distinct from those on mammary epithelial differentiation.

DISCUSSION

The data presented in this report demonstrate that expression of the novel SNF1-related serine/threonine kinase, *Hunk*, in the mammary gland is: tightly regulated during mammary development with a transient peak during early pregnancy; rapidly and synergistically induced in response to estradiol and progesterone; and spatially restricted within a subset of mammary epithelial cells throughout postnatal development. These data suggest a role for Hunk in mammary development, particularly with respect to pregnancy-induced changes in the mammary gland. Consistent with this hypothesis, misexpression of Hunk in the mammary gland disrupts normal lobuloalveolar development during pregnancy and lactation. Specifically, dysregulated Hunk expression results in decreased epithelial cell proliferation exclusively during mid-pregnancy as well as impaired alveolar cell differentiation throughout pregnancy and lactation. Together, these data suggest that Hunk may contribute to pregnancy-induced changes in the mammary gland, and that Hunk may play a role in the response of the mammary epithelium to ovarian hormones.

Although *Hunk* mRNA expression levels are markedly upregulated during early pregnancy, a developmental stage that is characterized by rapid alveolar cell proliferation, multiple lines of evidence suggest that *Hunk* expression is not simply a correlate of proliferation. For instance, the temporal profile of *Hunk* expression in the mammary gland during development is distinct from that of bona fide markers of proliferation such as cyclin A, cyclin D1, PCNA and PLK (Chodosh et al., 2000; Master et al., unpublished). Specifically, the upregulation of *Hunk* expression in the mammary gland is confined to early pregnancy, whereas the above proliferation markers are not only upregulated during early pregnancy, but also during mid-pregnancy as well as puberty. Moreover, *Hunk* is not preferentially expressed in proliferative as compared to non-proliferative compartments in the mammary gland (i.e. terminal end buds versus ducts during puberty, alveoli versus ducts during early pregnancy). Finally, analysis of actively growing versus confluent or serum-starved mammary epithelial cells reveals no difference in *Hunk* mRNA levels (H. P. G., unpublished). These observations suggest that *Hunk* expression does not simply reflect the proliferative state of the mammary epithelium, but rather may reflect other developmental pathways or events in the mammary gland.

Hunk upregulation in the mammary gland during early pregnancy is transient. This observation raises the intriguing possibility that the tightly regulated pattern of *Hunk* expression during pregnancy is required for normal lobuloalveolar development. We have tested this hypothesis by misexpressing *Hunk* in the mammary glands of transgenic mice. Forced overexpression of an MMTV-*Hunk* transgene in the mammary epithelium throughout postnatal development results in a defect in lobuloalveolar development with molecular abnormalities first discernible during early pregnancy, cellular abnormalities discernible during mid-pregnancy and morphological abnormalities discernible late in pregnancy. Specifically, Hunk overexpression results in a defect in epithelial proliferation that is restricted to mid-pregnancy and a defect in differentiation that is manifest throughout the developmental interval spanning day 6.5 of pregnancy to day 2 of lactation. In contrast, forced overexpression of *Hunk* in nulliparous animals has no obvious effect on patterns of proliferation or differentiation, or on the morphology of the mammary epithelial tree. Together, our findings suggest the possibility that the defects observed in lobuloalveolar development in MHK3 mice are due to the failure to downregulate Hunk expression during mid-pregnancy, rather than to Hunk overexpression per se.

Our finding that Hunk overexpression inhibits alveolar proliferation during mid-pregnancy was surprising given that *Hunk* is normally upregulated in the mammary gland during early pregnancy – the stage of pregnancy associated with maximum alveolar proliferation. This suggests either that the normal role of Hunk may be to negatively regulate mammary epithelial proliferation during pregnancy, or that the inhibitory effect of Hunk on proliferation at day 12.5 of pregnancy is a consequence of overexpression during a developmental stage at which Hunk is normally downregulated. Alternatively, the developmental profile of endogenous Hunk activity may be different from that of steady-state levels of *Hunk* mRNA. Interestingly, the human SNF1-related kinase, C-TAK1/KP78/MARK3, has been shown to phosphorylate and inactivate Cdc25c, thereby preventing activation of Cdc2 and presumably inhibiting entry of cells into mitosis (Peng et al., 1997, 1998). Consistent with this hypothesis, expression of KP78 protein has been reported to be downregulated in adenocarcinomas of the pancreas (Parsa, 1988). Thus, there is precedent for the negative regulation of cellular proliferation by mammalian SNF1 family members. Whether Hunk interacts with Cdc25c, negatively regulates cellular proliferation when expressed at physiological levels or is altered in human malignancies is unknown.

We have demonstrated defects in both mammary epithelial

proliferation and differentiation in MHK3 animals during pregnancy. For example, the lower total RNA yield obtained from transgenic glands as compared with wild-type glands during late pregnancy and lactation probably reflects, in part, the reduced epithelial cell content of MHK3 transgenic glands, since the increase in total RNA present in the mammary gland during lobuloalveolar development is a result both of increases in epithelial cell number and increases in expression of milk protein genes on a per-cell basis (Fig. 5B). As such, we initially considered the possibility that the decreased expression of markers for mammary epithelial differentiation observed in MHK3 animals during pregnancy and lactation is a consequence of the decreased alveolar proliferation evident in MHK3 mice at day 12.5 of pregnancy, and the resulting decrease in epithelial cell mass. However, several lines of evidence indicate that the abnormalities in mammary epithelial differentiation that we have described in MHK3 animals cannot be explained by a decrease in epithelial cell mass. First, the fact that defects in alveolar differentiation in MHK3 animals actually precede the reduction in epithelial proliferation that occurs at day 12.5 strongly argues that defects in differentiation cannot solely be a consequence of defects in proliferation. In addition, RNA extracted from a mammary gland composed of a smaller number of appropriately differentiated epithelial cells would be predicted to give rise to a normal distribution of milk protein gene expression (i.e. early versus late), and to normal levels of expression of milk protein genes when normalized to epithelial cell content. In contrast, our observations indicate that both the level and the composition of milk protein RNA produced by the mammary glands of MHK3 animals during pregnancy and lactation is abnormal even after controlling for differences in epithelial content between wild-type and transgenic glands. Consistent with this conclusion, the morphology of the alveolar epithelial cells present in the mammary glands of MHK3 animals at day 18.5 of pregnancy is less differentiated compared with those present in their wild-type counterparts. Thus, we conclude that the reduced expression of differentiation markers in MHK3 transgenic glands reflects the less differentiated state of the mammary epithelial cells present, rather than a reduced number of appropriately differentiated mammary epithelial cells. As such, our data indicate that the defects in differentiation that occur in MHK3 animals as a consequence of Hunk overexpression are separable from and, at least in part, independent of the defects in proliferation that occur in these animals.

With regard to the analysis presented in this manuscript, it is important to note that during pregnancy and lactation, a similar magnitude of reduction in the expression of differentiation markers was observed in the mammary glands of MHK3 animals compared with wild-type animals regardless of whether levels of expression of milk protein genes were normalized to β-actin or to the epithelial cell marker, cytokeratin 18 (Fig. 7 and data not shown). That is, when normalized to β-actin expression, cytokeratin 18 expression levels do not differ between MHK3 transgenic animals and wild-type animals at any stage of lobuloalveolar development. Presumably, this reflects the fact that mammary epithelial cells contribute the vast majority of RNA to the total RNA pool during pregnancy and lactation, an observation that explains why cytokeratin 18 levels show little change during pregnancy when normalized to β-actin expression. Thus, normalizing mRNA expression levels to β-actin mRNA levels itself effectively controls for the decreases in epithelial cell content that occur in MHK3 animals.

Surprisingly, lactoferrin expression in the mammary glands of both nulliparous and lactating mice was elevated in Hunk-overexpressing MHK3 animals compared either with wild-type animals or with non-expressing MHK3 transgenic animals. This observation suggests that while Hunk overexpression may inhibit mammary epithelial differentiation globally during pregnancy and lactation, the effects of Hunk overexpression on lactoferrin expression may be more specific. In support of this hypothesis, we have compared gene expression patterns in wild-type and MHK3 nulliparous transgenic glands using oligonucleotide-based cDNA microarrays. These microarray studies revealed that of the approx. 5500 genes analyzed, the gene for lactoferrin is one of only 16 genes whose expression changes by more than 2.5-fold in transgenic when compared with wild-type glands (H. P. G., unpublished). As noted above, the mammary glands of nulliparous MHK3 animals are morphologically indistinguishable from those of wild-type littermates. Thus, our findings indicate that the effects of Hunk overexpression on lactoferrin gene regulation are relatively specific, and are unlikely to be secondary to marked abnormalities in mammary gland morphology or to global changes in gene expression. Since lactoferrin expression in several tissues has been shown to be regulated by 17β-estradiol, EGF, protein kinase C and cAMP-mediated pathways (Teng, 1995), the specific upregulation of lactoferrin expression in MHK3 transgenic mice may provide a clue to signal transduction pathways in which Hunk may be involved.

Hunk is expressed in a heterogeneous, epithelial-specific manner throughout postnatal mammary development. This heterogeneous expression pattern is particularly striking in the terminal end bud during puberty and throughout the mammary epithelium during pregnancy, and suggests that *Hunk* may be a marker for a previously undescribed subtype of mammary epithelial cell or may be a marker for a particular cellular state. An intriguing hypothesis regarding the heterogeneous pattern of *Hunk* expression in both developing alveolar buds and epithelial ducts during pregnancy is that this heterogeneity may reflect the differing ability of mammary epithelial cells to respond to ovarian hormones. Testing this hypothesis will require the ability to colocalize the expression of endogenous Hunk protein with steroid hormone receptors and other markers of hormone responsiveness.

Finally, we have demonstrated that treatment of mice with 17β-estradiol and progesterone results in the rapid and synergistic upregulation of *Hunk* expression in the mammary gland. These findings suggest that the upregulation of *Hunk* expression in response to hormones is not a consequence of the marked changes in epithelial differentiation or epithelial cell number that occur either during early pregnancy or in response to the chronic administration of 17β-estradiol and progesterone. Interestingly, unlike the effect of steroid hormones on *Hunk* expression in the mammary gland, treatment of mice with 17β-estradiol either alone or in combination with progesterone results in downregulation of *Hunk* expression in the uterus. The opposing effects of combined estradiol and progesterone treatment on *Hunk* expression in the mammary gland and uterus is reminiscent

of the dichotomous effects of these hormones on epithelial proliferation in these tissues. As such, our finding that the effect of steroid hormones on *Hunk* expression in the mammary gland and uterus parallels the dichotomous response of these tissues to estradiol and progesterone provides additional support for the hypothesis that Hunk may be a downstream effector of estrogen and progesterone, and suggests a potential explanation for the dichotomous response of these tissues to steroid hormones.

In aggregate, our findings raise the possibility that the upregulation of *Hunk* expression in the mammary gland by estradiol and progesterone may contribute to changes in the mammary epithelium that occur during pregnancy by mediating the effects of steroid hormones. Ultimately, elucidating the mechanisms by which hormones regulate *Hunk* expression, and by which Hunk may regulate mammary epithelial proliferation and differentiation, may yield insights into the complex role which hormones play in mammary development and carcinogenesis.

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The CaM Kinase, *Pnck*, Is Spatially and Temporally Regulated during Murine Mammary Gland Development and May Identify an Epithelial Cell Subtype Involved in Breast Cancer[1]

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ABSTRACT

While screening for protein kinases expressed in the murine mammary gland, we identified previously a Ca^{2+}/calmodulin-dependent kinase, *Pnck*, that is most closely related to CaMKI. In this report, we show that *Pnck* is temporally regulated during murine mammary development with highest levels of expression observed late in pregnancy, concomitant with the decreased cellular proliferation and terminal differentiation of the mammary epithelium. Consistent with this finding, *Pnck* is up-regulated in confluent mammary epithelial cells and is down-regulated as serum-starved cells are stimulated to reenter the cell cycle. In the mammary gland, *Pnck* is expressed in an epithelial-specific and markedly heterogeneous manner, suggesting that the expression of this kinase may be restricted to a particular mammary epithelial cell type. Potentially related to its heterogeneous *in vivo* expression pattern, *Pnck* expression is oncogene-associated in murine epithelial cell lines derived from mammary tumors arising in different transgenic mouse models of breast cancer; cell lines derived from mammary tumors initiated by c-*myc* or *int-2/Fgf3* express *Pnck*, whereas cell lines initiated by *neu* or H-*ras* do not. In an analogous manner, expression of the human homologue of *Pnck* is restricted to a subset of human breast cancer cell lines. Moreover, *PNCK* was found to be highly overexpressed in a subset of human primary human breast cancers compared with benign mammary tissue. Together, our data suggest that Pnck may play a role in mammary development, and that expression of this kinase may be restricted to a mammary epithelial cell type that is transformed in a subset of human breast cancers.

INTRODUCTION

A woman's lifetime risk of developing breast cancer is intrinsically related to reproductive events, particularly those that affect the differentiated state of the breast. Results from both human epidemiology and animal model systems indicate that an early first full-term pregnancy results in a permanent change in the breast that confers a decreased risk for the subsequent development of breast cancer (1–4). The findings that aborted pregnancies, the majority of which occur prior to the third trimester, are not protective against breast cancer and that lactation has only a minimal protective effect compared with full-term pregnancy suggest that parity-induced protection against breast cancer results from physiological changes that occur late in pregnancy (5, 6). As a consequence, the protective effect of parity has been hypothesized to result from the impact of terminal differentiation on the susceptibility of the mammary epithelium to carcinogenesis (2, 3). Nevertheless, the molecular and cellular basis for this phenomenon is unknown. As such, understanding the developmental changes that occur in the breast late in pregnancy is essential for understanding the protected state of the breast associated with parity.

In an attempt to better understand the relationship between development and carcinogenesis in the breast, we previously carried out a screen designed to identify protein kinases that are expressed in the murine mammary gland during development and in mammary tumor cell lines (7–10). This resulted in the identification of the novel serine/threonine kinase, *Pnck*,[3] so named to reflect its temporally and spatially regulated pattern of expression in the mammary gland as described in this report. *Pnck* is a member of the CaM-dependent family of protein kinases and is most closely related to CaMKI (9). However, no significant homology is detected between Pnck and members of the CaM kinase family outside of the highly conserved catalytic and regulatory domains, suggesting that Pnck may have functions unique to this family of molecules.

Ca^{2+} is a key intracellular signaling molecule that exerts some of its effects by binding to calmodulin and activating CaM kinases. Calmodulin, in turn, has been implicated in development. For example, point mutations in the *Drosophila* calmodulin gene result in defects in development including pupal lethality and ectopic wing vein formation (11). Furthermore, calmodulin expression is regulated during cardiac development, and overexpression of calmodulin in murine cardiomyocytes results in cardiomyocyte hypertrophy (12). Like calmodulin, CaM kinases have been proposed to play diverse roles in development including CaMKIV in T-cell maturation and CaMKII in cell cycle regulation (13–16). However, developmental roles for multifunctional CaM kinases, including CaMKI, have not been defined.

We previously characterized the temporal and spatial patterns of *Pnck* expression during murine development (9). In murine embryos, *Pnck* expression is highest in developing brain, bone, and gastrointestinal tract. In adult mice, high levels of *Pnck* expression are found in the brain, uterus, ovary, and testis. Interestingly, within several tissues *Pnck* expression is limited to particular epithelial or stromal compartments, and within these compartments, *Pnck* expression is further restricted to a subset of cells (9). As such, the tissue-specific and spatially restricted patterns of *Pnck* expression suggest that this kinase may be involved in a variety of developmental processes.

In this report, we demonstrate that the CaM kinase, *Pnck*, is spatially and temporally regulated during murine mammary development with highest levels of expression observed late in pregnancy as alveolar epithelial cells exit the cell cycle and undergo terminal differentiation. Potentially related to this temporal pattern of expression, *Pnck* is up-regulated in confluent mammary epithelial cells and down-regulated as serum-starved cells are stimulated to reenter the cell cycle. We further show that *Pnck* expression in the mammary gland is restricted to a subset of epithelial cells during development and that *Pnck* is expressed in an oncogene-associated manner in cell

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[3] The abbreviations used are: Pnck, pregnancy up-regulated nonubiquitous CaM kinase; CaM, Ca^{2+}/calmodulin; MMTV, mouse mammary tumor virus.

lines derived from murine mammary tumors with defined initiating events. Similarly, expression of the human homologue of *Pnck* is restricted to a subset of human breast tumor cell lines and is highly overexpressed in a subset of primary human breast cancers. Taken together, our data suggest that *Pnck* may be expressed within a mammary epithelial cell type that is involved in differentiation as well as transformation.

MATERIALS AND METHODS

Animal and Tissue Preparation. FVB mice were housed under barrier conditions with a 12-h light/dark cycle. After sacrifice at the indicated developmental time points, the #3, 4, and 5 mammary glands were harvested. For RNA analysis, the lymph node embedded in mammary gland #4 was removed prior to harvest. Timed matings were set up such that all mice were sacrificed at ~16 weeks of age for comparison to adult nulliparous females. Day 0.5 postcoitus was defined as noon of the day on which a vaginal plug was observed. Time points at day 2 and day 7 of regression were obtained after removing pups at day 9 of lactation. Time points at day 28 of regression were obtained after 21 days of lactation. Tissues from 10 to 20 mice were pooled for each developmental time point. Tissues used for RNA analysis were snap frozen on dry ice. Tissues used for *in situ* hybridization analysis were embedded in OCT compound.

Tissue Culture. Murine cells were cultured in DMEM medium supplemented with 10% bovine calf serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Human cell line lines were cultured in the same medium with the addition of 5 μg/ml insulin. Transformed murine mammary epithelial cell lines were derived from tumors or hyperplastic lesions that arose in transgenic mice engineered to express different oncogenes under the control of the MMTV long terminal repeat. Cell lines from MMTV-c-*myc*, MMTV-*int-2/Fgf3*, MMTV-*neu*/NT, or MMTV-H-*ras* transgenic mice have been described previously (17). NIH 3T3, NMuMG, and CL-S1 murine cells, as well as human breast tumor cell lines, were obtained from American Type Culture Cells. HC11 cells were the kind gift of Jeff Rosen (Baylor College of Medicine, Houston, TX).

Actively growing cells were harvested at ~70% confluence. Confluent cells were refed daily and harvested 3 days after confluence. For serum starvation experiments, subconfluent cells were maintained in 0.1% serum for 2 days prior to refeeding in 10% bovine calf serum and harvested at the indicated time points.

RNA Analysis. RNA was prepared by homogenization of snap-frozen tissue samples or tissue culture cells in guanidinium isothiocyanate supplemented with 7 μl/ml 2-mercaptoethanol, followed by ultracentrifugation through cesium chloride as described previously (18, 19). Poly(A)$^+$ RNA was selected using oligo(dT) cellulose (Pharmacia). For Northern hybridization analysis, RNA was separated on a 1% LE agarose gel and passively transferred to a Gene Screen membrane (DuPont NEN). Hybridization was performed as described using a random primed, ^{32}P-labeled cDNA probe encompassing nucleotides 1355–1529 of c-*myc* (GenBank accession no. X01023), nucleotides 589-1287 of *cytokeratin 18* (GenBank accession no. M11686), or a 1.2-kb fragment containing the entire open reading frame of *cyclin D3* (19). RNase protection analysis was performed as described (19). Body-labeled antisense riboprobes were generated using [α-^{32}P]UTP and the Promega *in vitro* transcription system with T7 polymerase in combination with linearized plasmids containing nucleotides 1142–1241 of β-*actin* (GenBank accession no. X03672), nucleotides 911-1056 of *Gapdh* (GenBank accession no. M32599), nucleotides 1321–1509 of murine *Pnck* (GenBank accession no. AF181984), or a region of human *PNCK* corresponding to nucleotides 538–842 of murine *Pnck*. Riboprobes were hybridized with RNA samples overnight at 58°C in 50% formamide/100 mM PIPES (pH 6.7). Hybridized samples were digested with RNase A and T1, purified, electrophoresed on a 6% denaturing polyacrylamide gel, and subjected to autoradiography (XAR-5). β-*actin* or *Gapdh* antisense riboprobes were added to each reaction as an internal control. As a negative control, riboprobes were hybridized with tRNA and processed in parallel.

***In Situ* Hybridization.** *In situ* hybridization was performed as described (19). Antisense and sense riboprobes were synthesized with the Promega *in vitro* transcription system using [^{35}S]UTP and [^{35}S]CTP from the T7 and SP6 RNA polymerase promoters of a PCR template containing sequences corresponding to nucleotides 1135–1509 of *Pnck*. Exposure times were 7 weeks in all cases.

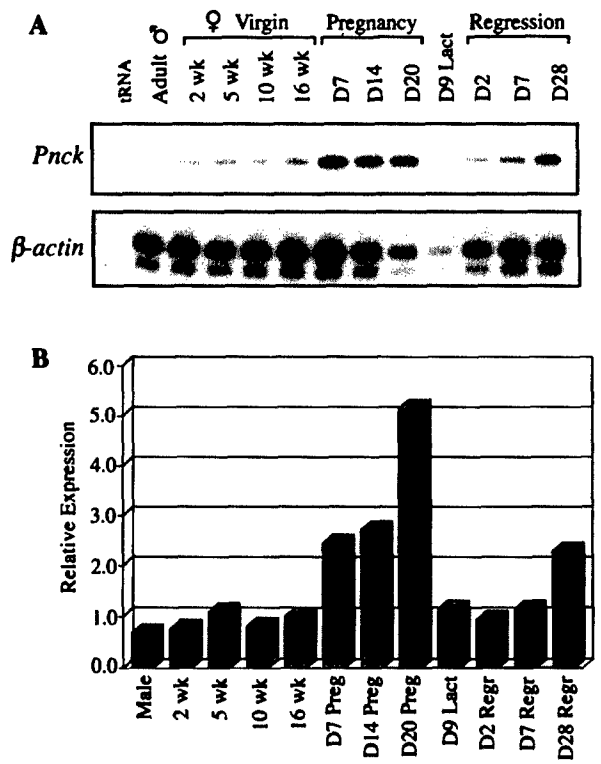

Fig. 1. Temporal regulation of *Pnck* expression during murine mammary gland development. *A*, RNase protection analysis of *Pnck* mRNA expression during postnatal murine mammary gland development. Forty μg of total RNA isolated from mammary glands at the indicated developmental time points were hybridized to ^{32}P-labeled antisense riboprobes specific for the 3′ untranslated region of *Pnck* or for β-*actin*. *B*, phosphorimager quantitation of RNase protection analysis in *A*. *Pnck* expression was quantitated and normalized to β-*actin* expression to correct for dilutional effects of large scale increases in milk protein gene expression during late pregnancy and lactation. Expression levels are shown relative to 16-week-old adult virgin animals.

RESULTS

Temporal Pattern of *Pnck* Expression during Mammary Development. In the course of screening for protein kinases with a potential role in mammary development and carcinogenesis, we isolated a CaM kinase family member, *Pnck*, from the mammary glands of mice undergoing early postlactational involution (7, 9). To begin to investigate the potential role of *Pnck* in mammary development, we examined the temporal profile of *Pnck* expression during the postnatal development of the murine mammary gland (Fig. 1). *Pnck* expression was normalized to β-*actin* expression to control for dilutional effects resulting from the massive increases in milk protein gene expression that occur during late pregnancy and lactation (Refs. 7, 19, and 20; Fig. 1*B*). As verified by quantitative *in situ* hybridization analysis, normalization of gene expression to β-*actin* expression provides a more accurate assessment of changes in gene expression on a per cell basis than normalization solely to the amount of RNA assayed (Fig. 2).[4]

Pnck mRNA expression levels were found to be low and relatively constant in nulliparous animals between 2 and 16 weeks of age, a period that encompasses ductal morphogenesis (Fig. 1). In contrast, a 2-fold up-regulation of *Pnck* expression was observed early in pregnancy as compared with age-matched nulliparous animals. *Pnck* ex-

[4] J. Hartman, unpublished results.

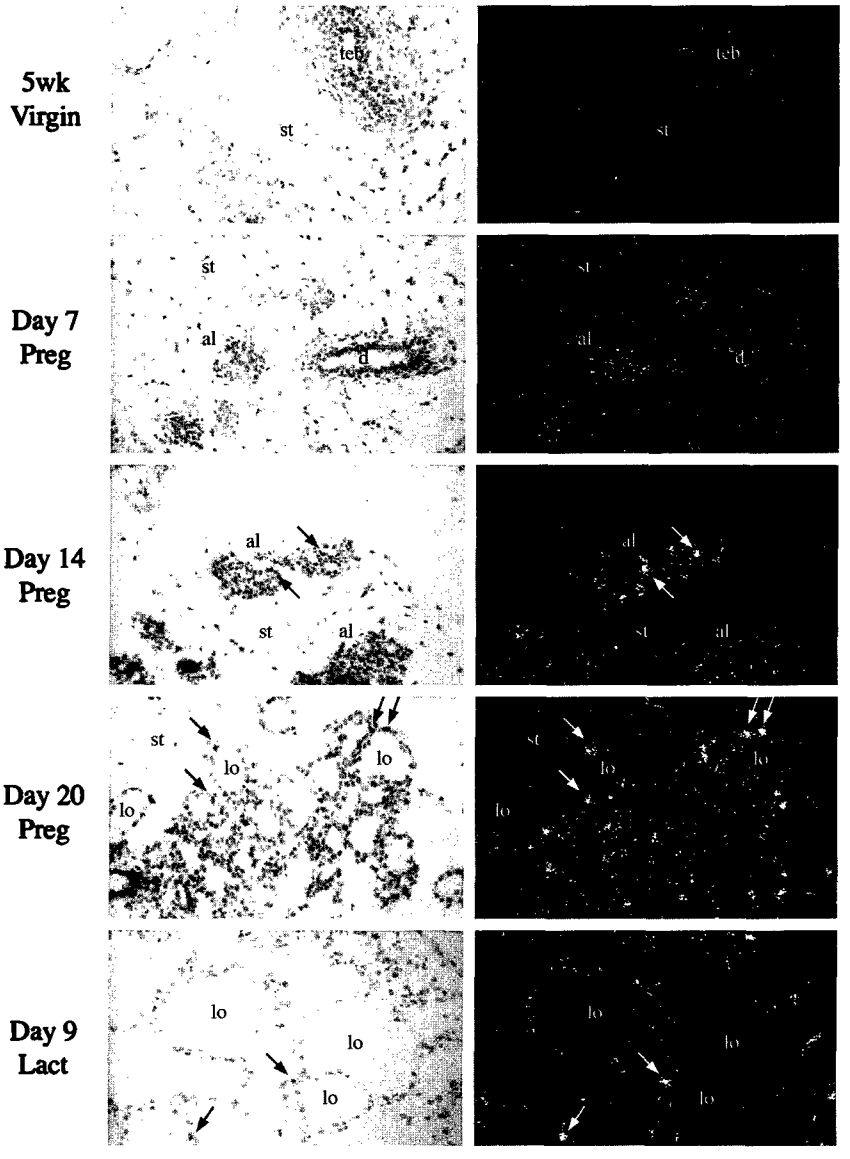

Fig. 2. Spatial regulation of *Pnck* expression in the mammary gland. *In situ* hybridization analysis of *Pnck* expression in the mammary gland during development. Bright-field (*left panels*) and dark-field (*right panels*) photomicrographs of mammary gland sections hybridized with an ^{35}S-labeled *Pnck*-specific antisense riboprobe. No signal over background was detected in serial sections hybridized with the corresponding sense probe. Exposure times were identical for all dark-field photomicrographs to illustrate changes in *Pnck* expression during pregnancy and lactation. *Arrows, Pnck*-expressing epithelial cells. *al,* alveoli; *d,* duct; *lo,* alveolar lobule; *st,* adipose stroma; *teb,* terminal end bud. ×300.

pression remained elevated during mid-pregnancy and attained maximal levels of expression (5-fold) late in pregnancy, concomitant with the cessation of proliferation and terminal differentiation of the alveolar epithelium. *Pnck* expression levels returned to baseline during lactation and early postlactational regression. Notably, steady-state levels of *Pnck* mRNA were higher in the mammary glands of parous animals after 4 weeks of postlactational involution as compared with age-matched nulliparous animals.

Heterogeneous Expression of *Pnck* in the Mammary Epithelium. To determine whether pregnancy-induced changes in *Pnck* mRNA expression levels represent global changes in expression throughout the mammary gland or changes within a subpopulation of cells, *in situ* hybridization analysis was performed (Fig. 2). Consistent with our RNase protection results, *in situ* hybridization confirmed that *Pnck* expression peaks late in pregnancy. Furthermore, throughout postnatal development *Pnck* expression was detected only in the mammary epithelium and was strikingly heterogeneous during pregnancy, with highly expressing cells located adjacent to cells in which *Pnck* expression was low or undetectable. The spatial heterogeneity of *Pnck* expression was most marked during late pregnancy, at which time only a small fraction of epithelial cells was observed to express *Pnck* at high levels. The heterogeneous spatial pattern of *Pnck* expression differs from that observed for other protein kinases that we have examined, as well as for genes such as *cytokeratin 18, Gapdh,* and *β-actin* (7).

***Pnck* Expression *in Vitro*.** The observation that *Pnck* expression peaks late in pregnancy as alveolar epithelial cells exit the cell cycle and undergo terminal differentiation suggested that *Pnck* mRNA expression may be inversely related to mammary epithelial proliferation. To investigate this possibility, *Pnck* mRNA levels were analyzed in actively proliferating or confluent mammary epithelial cell lines (Fig. 3*A*). This analysis revealed that steady-state levels of *Pnck* mRNA were an average of 3.7-fold higher in confluent cells compared with actively proliferating cells (Student's t test, $P < 0.01$). To distinguish whether this increase in *Pnck* expression was attributable to decreased proliferation or to the establishment of cell-cell contacts in confluent cells, *Pnck* expression levels were analyzed in subconfluent serum-starved mammary epithelial cells as they reentered the cell cycle after refeeding (Fig. 3*B*). Consistent with the up-regulation of *Pnck* expression observed in confluent cells, refeeding of serum-starved cells resulted in a rapid decrease in *Pnck* expression that began within 1 h and reached a nadir at 4 h after refeeding. Identical results were observed in a second mammary epithelial cell line (data not shown).

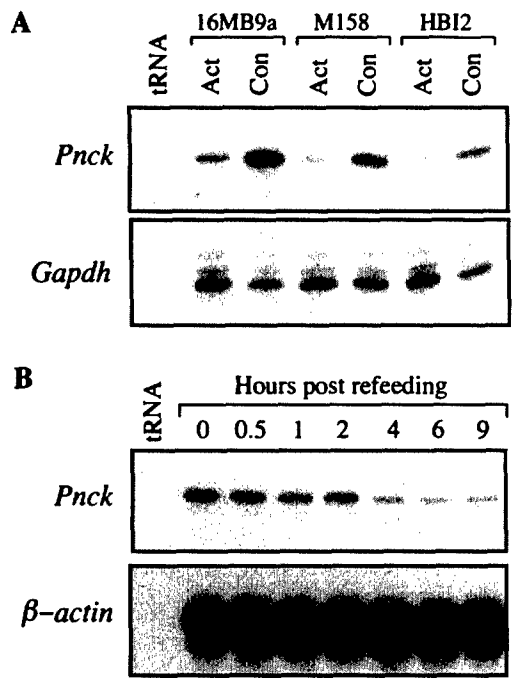

Fig. 3. Proliferation-dependent expression of *Pnck*. *A*, RNase protection analysis of *Pnck* expression in actively growing *versus* confluent cells. ^{32}P-labeled antisense riboprobes specific for *Pnck* or *Gapdh* were hybridized with 30 μg of total RNA isolated from the indicated cell lines while either actively growing (*Act*) or 3 days after confluence (*Con*). *B*, RNase protection analysis of *Pnck* expression in serum-starved 16MB9a cells at the indicated times after refeeding. Thirty μg of total RNA isolated from cells at each time point were hybridized with ^{32}P-labeled antisense riboprobes specific for *Pnck* or *β-actin*.

***Pnck* Expression in Transgenic Mammary Tumor Cell Lines.** To begin to examine the potential role of *Pnck* in mammary tumorigenesis and to investigate the hypothesis that *Pnck* is expressed in an epithelial cell subtype in the mammary gland, *Pnck* mRNA expression was examined in a panel of mammary epithelial cell lines derived from independent adenocarcinomas arising in MMTV transgenic mice expressing either the *neu/NT*, c-*myc*, H-*ras*, or *int-2/Fgf3* oncogenes in the mammary epithelium (Ref. 17; Fig. 4). All cell lines were proliferating at similar rates when harvested as evidenced by their similar levels of *cyclin D3* mRNA expression. *Pnck* expression was not detected in NIH 3T3 fibroblasts, consistent with its epithelial-specific pattern of expression in the mammary gland *in vivo*. Interestingly, *Pnck* was expressed in all seven cell lines derived from mammary tumors or hyperplasias arising in MMTV-c-*myc* and MMTV-*int-2/Fgf3* transgenic mice. In contrast, *Pnck* expression was undetectable in the eight cell lines derived from mammary tumors arising in MMTV-*neu* and MMTV-H-*ras* transgenic mice, despite the fact that RNase protection analysis was performed using poly(A)$^+$ RNA. Similarly, *Pnck* expression was not detected in any of the three nontransformed mammary epithelial cell lines examined including confluent or differentiating HC11 cells (Fig. 4 and data not shown). Analysis of the expression of 40 other protein kinases identified in our screen indicated that this particular oncogene-associated pattern of expression is unique to *Pnck* (7, 20). Of note, *Pnck* expression did not appear to correlate with absolute levels of either endogenous c-*myc* or c-*myc* transgene expression (Fig. 4). This observation suggests that the oncogene-restricted pattern of *Pnck* expression may not be the result of c-*myc*-induced activation of *Pnck* transcription.

***PNCK* Expression in Human Breast Tumor Cell Lines and Primary Breast Tumors.** To further investigate the potential involvement of *Pnck*, or a cell type in which *Pnck* is expressed, in mammary carcinogenesis, we determined *PNCK* expression levels in a panel of human breast cancer cell lines (Fig. 5). Similar to the wide range of *Pnck* expression observed in the murine mammary epithelium and in murine mammary tumor cell lines, *PNCK* expression was detected in only a subset of human breast tumor cell lines. High levels of *PNCK* expression were observed in 3 of 18 breast tumor cell lines. Eight cell lines expressed low but detectable levels of *PNCK*, whereas no *PNCK* expression was detected in the remaining seven cell lines. As in murine mammary tumor cell lines, *PNCK* expression levels did not correlate with c-*MYC* expression (data not shown).

The heterogeneous pattern of *Pnck* expression observed *in vitro* in both murine and human breast tumor cell lines suggested the possibility that *PNCK*-expressing and *PNCK*-nonexpressing breast tumor types might exist. To test this hypothesis directly, we used RNase protection analysis to quantitate *PNCK mRNA* expression levels in a panel of 23 primary human breast tumors. The resulting expression levels were compared with *PNCK* expression levels in 12 benign breast tissue samples (Fig. 6*A*). This analysis revealed two interesting aspects of the pattern of *PNCK* expression in breast tumors compared with benign tissue: (*a*) *PNCK* is expressed at significantly higher levels in breast tumors compared with benign tissue; and (*b*) *PNCK* expression in human tumors is markedly heterogeneous.

Statistical analysis of the above *PNCK* expression levels indicated that when normalized to *β-actin* expression, *PNCK* expression in human primary breast cancers is ~5-fold higher than in benign breast tissue (Student's *t* test, $P = 0.01$; Fig. 6*B*). However, because *PncK* expression in the mammary gland is epithelial specific and because tumors typically have a higher epithelial content than benign breast tissue, we also normalized *PNCK* expression to expression of the epithelial-specific marker, *cytokeratin 18, (CK18)*, to control for the increased epithelial cell content of tumors (Fig. 6*B*). Strikingly, even after normalization to *CK18* expression, *PNCK* expression levels were

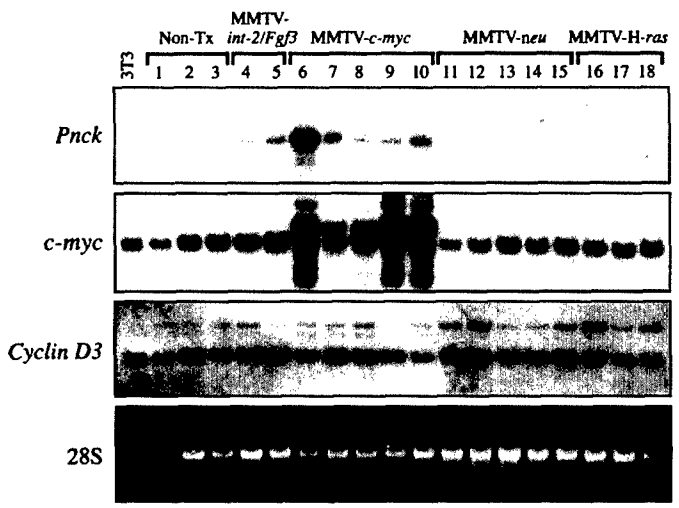

Fig. 4. *Pnck* expression in nontransformed and transformed murine mammary epithelial cell lines. Transformed cell lines were derived from mammary adenocarcinomas arising in MMTV transgenic mice expressing the *int-2/Fgf3*, c-*myc*, *neu*, or H-*ras* oncogenes in the mammary gland (17). RNase protection analysis was performed on 6 μg of poly(A)$^+$ RNA isolated from actively growing murine cell lines hybridized with a ^{32}P-labeled antisense riboprobe specific for the 3′ untranslated region of *Pnck* (*top panel*). Northern analysis was performed on 6 μg of poly(A)$^+$ RNA using ^{32}P-labeled cDNA probes specific for c-*myc* (*middle panel*) or *cyclin D3* (*bottom panel*). Note, the upper band observed in MMTV-c-*myc*-derived cell lines corresponds to *c-myc* transgene expression. The poly(A)$^+$ RNA beneath the 28S rRNA band is shown as a loading control. Cell lines are: NIH-3T3 fibroblast, nontransformed (*Non-Tx*): *Lane 1*, NMuMG, *Lane 2*, HC11, and *Lane 3*, CL-S1. MMTV-*int-2/Fgf3*: *Lane 4*, HBI2; and *Lane 5*, 1128. MMTV-*c-myc*: *Lane 6*, 8MA1a; *Lane 7*, MBp6; *Lane 8*, M1011; *Lane 9*, M158; and *Lane 10*, 16MB9a. MMTV-*neu*: *Lane 11*, SMF; *Lane 12*, NaF; *Lane 13*, NF639; *Lane 14*, NF11005; and *Lane 15*, NK-2. MMTV-H-*ras*: *Lane 16*, AC816; *Lane 17*, AC711; and *Lane 18*, AC236.

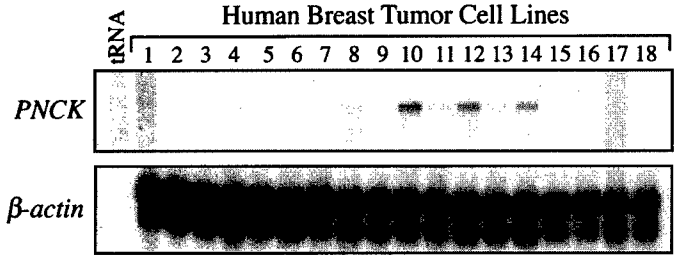

Fig. 5. *PNCK* is expressed in a subset of human breast tumor cell lines. RNase protection analysis of 30 μg of total RNA isolated from actively growing human breast tumor cell lines hybridized with a ^{32}P-labeled antisense riboprobe specific for *PNCK* or for *β-actin*. Cell lines are: *Lane 1,* 184B5; *Lane 2,* 2 MT-2; *Lane 3,* BT-20; *Lane 4,* BT-474; *Lane 5,* BT-549; *Lane 6,* HBL-100; *Lane 7,* MDA-MB-157; *Lane 8,* MDA-MB-231; *Lane 9,* MDA-MB-361; *Lane 10,* MDA-MB-435; *Lane 11,* MDA-MB-436; *Lane 12,* MDA-MB-453; *Lane 13,* MDA-MB-468; *Lane 14,* SK-BR-3; *Lane 15,* ZR-75-1; *Lane 16,* MCF-10; *Lane 17,* MCF-10A; and *Lane 18,* Hs 578T.

found to be three times higher in human primary breast tumors than in benign tissue (*t* test, $P = 0.039$).

Formally, the increase in *PNCK* expression levels in breast tumors compared with benign tissue could result either from increased expression among all tumors or from increased expression in a subset of tumors. In this regard, analysis of the distribution of *PNCK* expression among the 23 ductal carcinomas studied revealed a wide range of *PNCK* expression levels, in contrast to the relatively similar levels of *PNCK* expression observed among benign breast tissue samples. Notably, the mode for the benign and tumor distributions was the same (Fig. 6, *A* and *C*). Indeed, examination of the histogram representing *CK18*-normalized *PNCK* expression levels revealed that 8 of the 23 primary breast tumors analyzed express *PNCK* at levels greater than 3 SDs above the mean observed for benign samples (Fig. 6*C*). This difference is highly significant because no tumors would have been predicted to express *PNCK* at these levels if the distribution of *PNCK* expression in tumors was similar to that observed in benign tissues. Even more strikingly, four breast tumors were found to express *PNCK* at levels >10 SDs above the mean observed for benign tissues. Together, these data indicate that *PNCK* is overexpressed in human primary breast cancers compared with benign tissue, and that this observed increase is attributable to high levels of *PNCK* expression in a subset of breast tumors.

DISCUSSION

We have demonstrated that expression of the CaM kinase, *Pnck*, is temporally and spatially regulated in the murine mammary gland during postnatal development and that *Pnck* expression is restricted to a subset of mammary epithelial cells both *in vivo* and *in vitro*. Furthermore, our findings that *Pnck* is up-regulated in serum-starved and confluent cells suggest that the up-regulation of *Pnck* expression in the mammary gland late in pregnancy may be related to the decreased proliferation of mammary epithelial cells during this stage of development. We have shown that *Pnck* is expressed in an oncogene-associated manner in murine mammary tumor cell lines with defined genetic initiating events, and that *PNCK* expression is restricted to a subset of human breast tumor cell lines. Finally, we have demonstrated that *PNCK* is overexpressed in human primary breast cancers compared with benign breast tissue and that this overexpression is restricted to a subset of human breast tumors. In aggregate, our findings are consistent with the hypothesis that *PNCK* expression is restricted to a subset of ductal carcinomas in humans and suggest a role for *PNCK*, or a cell type that expresses *PNCK*, in mammary carcinogenesis. Our findings represent the first data implicating a CaM kinase in mammary development or carcinogenesis.

Both calmodulin and CaM-dependent kinases have been reported previously to be involved in cell cycle progression (21–26). Our data demonstrate that *Pnck* expression *in vitro* is inversely correlated with cellular proliferation. Specifically, decreasing the proliferative status of mammary epithelial cells *in vitro* resulted in increased *Pnck* expression. Interestingly, both the up-regulation of *Pnck* observed in confluent cells and the down-regulation of *Pnck* observed as serum-starved cells reenter the cell cycle are consistent with *Pnck* expression patterns in the mammary gland during late pregnancy. Although the up-regulation of *Pnck* observed during late pregnancy could simply be an effect of decreased epithelial proliferation, *Pnck* up-regulation could also be directly involved in inhibiting cellular proliferation or contributing to the exit of epithelial cells from the cell cycle prior to their terminal differentiation. Nevertheless, although a role for *Pnck* in cell cycle regulation is plausible, further work will be required to establish this relationship.

The markedly heterogeneous spatial expression pattern that we have observed for *Pnck* in the mammary gland is unusual compared with other genes that we have investigated (7).[4] Moreover, the observation that *Pnck* expression peaks late in pregnancy and remains heterogeneous throughout pregnancy and lactation distinguishes *Pnck* from milk protein genes and other markers of mammary epithelial differentiation (27). Although the expression patterns of milk protein genes such as *β-casein*, *WAP*, and *α-lactalbumin* are spatially heterogeneous during the developmental stages at which they are initially induced, each of these genes is expressed homogeneously throughout the mammary epithelium when their expression peaks during lactation. These data suggest that *Pnck* expression is not simply a marker for terminally differentiated mammary epithelial cells. Indeed, the down-regulation of *Pnck* expression in the mammary gland during lactation is consistent with a model in which this kinase plays a role in the process of differentiation but not in the maintenance of the differentiated state *per se*.

At least two hypotheses could account for the heterogeneous pattern of *Pnck* expression in mammary epithelial cells *in vivo* and *in vitro*: (*a*) only a small percentage of mammary epithelial cells may express *Pnck* at any given time, but all cells may express *Pnck* at some time. Such a model is consistent with genes whose expression is cell cycle regulated but is inconsistent with our finding that multiple mammary epithelial cell lines do not express *Pnck*; (*b*) alternatively, we favor the hypothesis that *Pnck* expression may identify an as yet undefined mammary epithelial cell type. This hypothesis is consistent with our findings that *Pnck* expression is detected within only a subset of cells in the mammary epithelium *in vivo*, as well as within a subset of murine breast cancer cell lines, human breast cancer cell lines, and primary human breast cancers. Nevertheless, this hypothesis does not rule out the possibility that *Pnck* expression may be modulated within expressing cell types or that *Pnck*-expressing cell types may only express *Pnck* during certain physiological states. In aggregate, the expression patterns for *Pnck* described in this report suggest that a *Pnck*-expressing cell type exists that may have unique properties with respect to mammary development and mammary epithelial transformation.

Potentially related to the expression of *Pnck* in a mammary epithelial cell subtype, *Pnck* expression was found to be restricted to cell lines derived from murine mammary tumors initiated by the oncogenes c-*myc* or *int-2/Fgf3* as compared with those initiated by an activated form of *neu* or by H-*ras*. Although such oncogene-associated patterns of expression are unusual, we and others have reported genes whose patterns of expression are the inverse of that observed for *Pnck* (7, 17, 28). Previous reports have demonstrated that murine mammary tumors induced by the expression of H-*ras*, c-*myc*, *neu*, or *int-2/Fgf3* each have histological patterns that are highly specific for

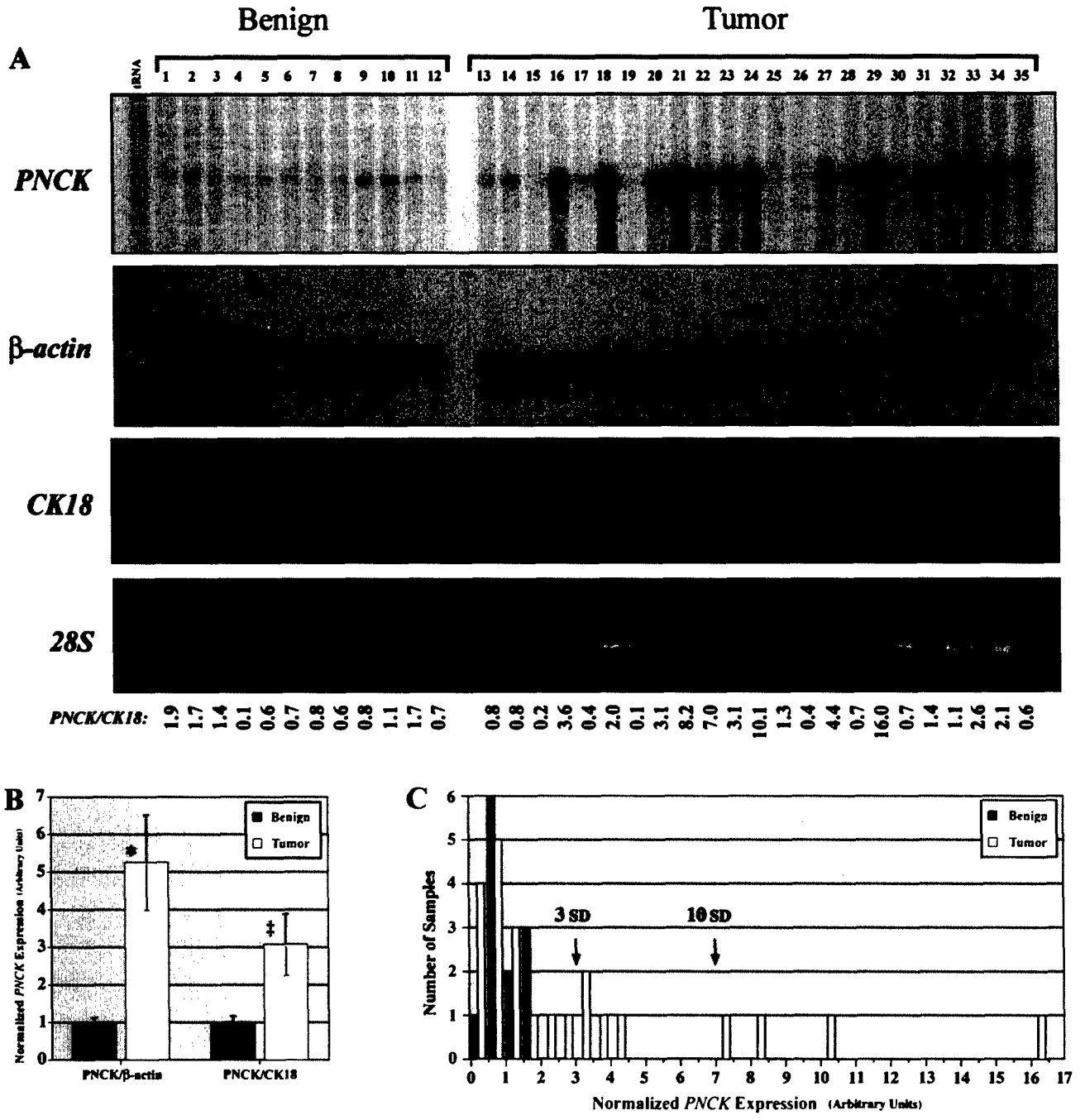

Fig. 6. *PNCK* is overexpressed in a subset of human primary breast tumors. RNA was isolated from 12 benign breast tissue samples and from 23 primary breast tumors obtained after surgery as indicated. *A*, RNase protection analysis was performed using 10 μg of total RNA hybridized with a ^{32}P-labeled antisense riboprobe specific for *PNCK* or for *β-actin* as indicated. Northern hybridization analysis was performed on the same RNA samples using 3 μg of total RNA hybridized with a ^{32}P-labeled cDNA probe specific for *cytokeratin 18 (CK18)*. The 28S rRNA band is shown as a control for equal RNA loading. *PNCK*, *β-actin*, and *CK18* expression levels were quantitated by phosphorimager analysis. *PNCK* expression levels normalized to *CK18* are shown for each sample. *B*, *PNCK* expression levels in breast tumors compared with benign tissue. *PNCK* expression levels for the samples shown in *A* were normalized either to *β-actin* or to *CK18*, as indicated. Normalized *PNCK* expression levels in benign tissues was set equal to 1.0. The means of each distribution are shown. *Bars*, SE. *, $P = 0.01$ for *PNCK/β-actin* expression in tumors compared with benign tissue. ‡, $P = 0.039$ for PNCK/*CK18* expression in tumors compared with benign tissue. *C*, histogram of individual *PNCK* expression levels normalized to *CK18* for primary breast tumors and benign breast tissue samples shown in *A*. *PNCK* and *cytokeratin 18* expression levels were quantitated by phosphorimager analysis. *PNCK* expression for each sample was normalized to *CK18* expression and the average expression in benign samples was set equal to 1.0. Values represent fold changes relative to the mean *PNCK/CK18* expression level observed for benign breast tissue. Bin sizes are 0.5 unit. Note that the mode for both the tumor and the benign samples is the same.

the inducing oncogene (29, 30). These morphological differences have been hypothesized to result either from the activation of unique downstream pathways or from the preferential transformation of different epithelial cell types by these oncogenes (17). Although c-*myc* or *int-2/Fgf3* could directly up-regulate *Pnck* expression, the lack of correlation between *Pnck* expression and c-*myc* expression in mammary tumor cell lines, along with the punctate expression of *Pnck in vivo*, raises the possibility that the oncogene-associated expression of *Pnck* may be more likely to result from the preferential transformation of a *Pnck*-expressing cell type by c-*myc*. Experiments are currently under way to directly address these and other potential explanations.

Superficially, our finding that one-third of human primary breast tumors overexpress *PNCK* compared with benign tissue seems inconsistent given the data presented in this report demonstrating an inverse correlation between *Pnck* expression and cellular proliferation. However, because negative regulators of the cell cycle are commonly up-regulated in tumors, it is possible that the observed up-regulation of *PNCK* in human tumors is a consequence of intact cell cycle checkpoints functioning to retard tumor growth. That is, if *PNCK* plays a negative role in cell cycle progression, the up-regulation of *PNCK* in breast tumors may be a result of the transformation process rather than an indication that PNCK plays a causal role in tumorigenesis.

We have hypothesized that *Pnck* expression is restricted to a mammary epithelial cell type that is transformed in a subset of breast cancers. This hypothesis is consistent with our findings that expression of the human homologue of *Pnck* is restricted to a subset of human breast tumor cell lines and is highly overexpressed in a subset of human breast tumors since these results could reflect the selection and enrichment of a particular epithelial cell type. Alternately, it is possible that *PNCK* expression in these cell lines and primary tumors does not correlate with a particular epithelial cell type or with a particular initiating genetic event. However, given the restricted pattern of *Pnck* expression in the mammary epithelium *in vivo,* along with the oncogene-associated pattern of *Pnck* expression in murine mammary tumor cell lines, we favor the hypothesis that selective expression of *PNCK* in human tumors is not random but rather reflects differences in the events that led to their transformation. Because tailoring specific therapeutic regimens to individual cancers bearing distinct molecular profiles may enhance the efficacy of breast cancer treatments, it will be important to evaluate the biological significance of the differential expression of *PNCK* in human breast cancers. For instance, it is possible that *PNCK*-overexpressing tumors may behave differently than other breast tumors and may thereby be associated with a different prognosis. Ultimately, the identification of either additional genes involved in breast cancer or genetic markers that identify different molecular subtypes of breast cancers will be invaluable both in improving our understanding of the pathogenesis of breast cancer and in promoting more effective treatments.

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DEPARTMENT OF THE ARMY
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 21 Feb 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

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